Aldehyde Adducts and Lung Injury

Muna Sapkota

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ALDEHYDE ADDUCTS AND LUNG INJURY

by

Muna Sapkota

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Environmental Health, Occupational Health & Toxicology Graduate Program

Under the Supervision of Professor Todd A Wyatt

University of Nebraska Medical Center

Omaha, Nebraska

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ALDEHYDE ADDUCTS AND LUNG INJURY

Muna Sapkota, Ph.D.
University of Nebraska, 2016
Supervisor: Todd. A. Wyatt, Ph.D.

Abstract

From the time of discovery, people have used both alcohol and tobacco in various forms all around the world. Alcohol abuse and cigarette smoking causes thousands of preventable deaths in the US each year. Several lung diseases such as COPD, asthma, respiratory infections, pneumonia, acute respiratory distress syndrome, cancer of neck, throat, mouth and lung are associated with smoking and drinking. Even though some studies have examined the role of alcohol and cigarette smoke in lung diseases, only a few studies have focused on the role of reactive aldehydes generated in the lungs of those who abuse alcohol and smoke cigarettes. In this study, we showed that reactive aldehydes formed in lung can attack protein and DNA to form various adducts. Malondialdehyde, a reactive aldehydes formed from both alcohol metabolism and cigarette smoking forms malondialdehyde deoxyguanosine adduct (M1dG) with guanine base of DNA. Our study showed that this adduct is formed in both human bronchial epithelial cells and in lungs of mice exposed to alcohol and cigarette smoke. In bronchial epithelial cells, M1dG adduct induced DNA damage. Our study also showed that use of dietary supplement such as diallyl-disulfide (DADS), a CYP2E1 inhibitor, significantly reduced DNA damage and M1dG adduct formation showing the important role of CYP2E1 in M1dG adducts formation. These findings suggest that CYP2E1 plays a pivotal role in alcohol-induced M1dG adducts, and the use of DADS as dietary supplement can reverse the effects of alcohol on M1dG formation.

With regard to protein adduction, our other study showed that reactive aldehydes acetaldehyde and malondialdehyde, when generated in lung in large concentrations, could form
hybrid adduct malondialdehyde-acetaldehyde adducts (MAA) to various biological proteins. Our study showed that this hybrid adduct, once formed, could induce inflammation in both cell and \textit{in vivo} model systems. MAA reduced macrophage phagocytic, superoxide ion and nitrite ion release functions. Also, MAA induced pro-inflammatory cytokines TNF alpha and IL-6 from macrophages. But in the absence of scavenger receptor A (SRA), previously known to bind MAA in bronchial epithelial cells, the effects of MAA were diminished; suggesting an important role for SRA in MAA-mediated changes to macrophage function. These results further support our previous finding that SRA is necessary for MAA binding.

In accordance with our \textit{in vitro} study, our mouse study results suggest that SRA is important for SPD-MAA induced lung injury. In the absence of SRA, there were significantly fewer total cells and neutrophils in the BAL fluid when mice were intranasally instilled with MAA for 3 weeks. In addition, there was less binding of MAA to lung epithelium and decreased neutrophil influx in the lung parenchyma of SRA KO mice. These data demonstrate that SRA plays an important role in SPD-MAA-induced inflammation in lung as evidenced by the diminished inflammation in the absence of this receptor.

Taken together, our data suggest that aldehyde adducts formation in lung is important and may provide an effective mechanism through which alcohol and cigarette smoke could initiate lung pathogenesis. Also, the role of CYP2E1 in lung aldehydes generation and SRA in MAA-mediated lung effects could be helpful in developing a novel treatment pathway for alcohol abusers who smoke cigarettes.
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<tr>
<td>AA</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>AcLDL</td>
<td>acetylated low-density lipoprotein</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate-activated protein</td>
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<tr>
<td>A2M</td>
<td>alpha 2 macroglobulin</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AODS</td>
<td>antioxidant defense system</td>
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<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>AUDs</td>
<td>alcohol use disorder</td>
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<tr>
<td>BAC</td>
<td>blood alcohol concentration</td>
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<tr>
<td>BAL</td>
<td>bronchoalveolar lavage fluid</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CSE</td>
<td>cigarette smoke extract</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>cytochrome P450 2E1</td>
</tr>
<tr>
<td>DADS</td>
<td>diallyl-disulfide</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ELE</td>
<td>epithelium lining environment</td>
</tr>
<tr>
<td>FEV</td>
<td>forced expiratory volume</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HBEC</td>
<td>human bronchial epithelial cells</td>
</tr>
<tr>
<td>HBSS</td>
<td>hanks balanced salt solution</td>
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<tr>
<td>4-HNE</td>
<td>4-hydroxynonenol</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
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</table>
IL-8     interleukin 8
JNK      c-Jun amino-terminal kinases
LDL      low-density lipoprotein
LPO      lipid peroxidation
MAA      malondialdehyde-acetaldehyde
MARCO    macrophage receptor with collagenous structure
MAPK     mitogen activated protein kinase
MIP2     macrophage inflammatory protein-2
M1dG     malondialdehyde deoxyguanamine
MMP-9    matrix metalloproteinase 9
NF-κB    nuclear factor kappa B
OTM      olive tail moment
OxLDL    oxidized low-density lipoprotein
PMs      peritoneal macrophages
PKC      protein kinase C
PM       particulate matter
ROS      reactive oxygen species
RNS      reactive nitrogen species
SPA      surfactant protein A
SPD      surfactant protein D
SRA      scavenger receptor A
TGF      transforming growth factor
TNF-α    tumor necrosis factor-alpha
SHS      second hand smoke
ETS      environmental tobacco smoke
WHO      world health organization
SIDS  sudden infant death syndrome
THS  third hand smoke
NIAAA  national institute of alcohol abuse and alcoholism
IARC  international agency for research on cancer
CHAPTER ONE:

1. Background

1.1 History of cigarette smoke

Within 150 years of Columbus encountering these “strange leaves”, tobacco was extensively used all around the world in various forms during different cultural or religious events (1). Initially, the fine ground form of tobacco was used as a mild analgesic and antiseptic to heal minor health illnesses such as cold, headache and eye problems. Chewing tobacco was recommended for oral ailments such as toothache, gum diseases and aches in the throat (2). Later, smoking of tobacco was introduced around the mid 17th century. Snuff held sway as popular in the 18th century, whereas cigar smoking was popular in the 19th century. It was believed that smoking tobacco would calm the nerves and relieve anxiety as well as improve body odor and prevent the plague (2). As smoking tobacco became popular over time, there was an increase in both the manufacture of cigarettes and the number of cigarette smokers. This led to the industrialization of tobacco production (1). Smoking among men was expanded due to free cigarettes being distributed to soldiers during World War II (3). People continued to smoke, which resulted in an increased trend in deaths from tobacco despite several studies showing the harmful effects of tobacco use (4).

1.2 Cigarette smoke and public health threat

During the initial days of discovery, tobacco was considered to be a magic cure for most ailments. Slowly, it was realized that tobacco was not a cure for all. An anonymous pamphlet titled “Work for Chimney-Sweeper” distributed in London around 1601 indicated tobacco as a poison. Tobacco use, either recreational or medicinal, was thought to weaken the body and was considered harmful (2). Still, smoking remained common in several public places such as theaters, restaurants and even airplane cabins while almost half of US adults continued to smoke cigarettes up to the 1960’s (5). Until the 19th century, tobacco was not even suspected as a cause
of lung tumors. But in the middle of the 20\textsuperscript{th} century, four distinct lines of evidence were proposed to establish cigarette smoking as a cause of lung cancer (population studies, animal experimentation, cellular pathology and identification of carcinogens in cigarette smoke)(6). Today, tobacco smoking is the leading cause of approximately 480,000 preventable deaths in the US annually. The economic burden of smoking is approximately $300 billion per year due to productivity loss and health care cost expenditures (7). Smoking is prevalent among males, adults aged between (25-44) years, persons with a high school education or less, and people living below the poverty level (8). Smoking rates are high among racial/ethnic minority groups and persons of lower socioeconomic status (SES) as a result of tobacco-control disparity (9). Smoking is now attributed to about 30\% of all cancers in the US. Lung cancer is the most common cause of smoking-related mortality in the US. The other smoking cancer risks are oral, pharyngeal and esophageal (10). The International Agency for Research on Cancer (IARC) has reported 55 carcinogens in cigarette smoke that have carcinogenic effects in laboratory animals or humans (11). Although the consumption of cigarette smoking is decreased in US, it is increasing in developing countries (12). The morbidity and mortality in these countries is likely to surpass that of developed countries in the coming next century due to the delayed health effects of smoking (13). Deaths from lung cancer, ischemic heart disease and chronic airway obstruction are attributed to smoking in adults. In addition to this, several hundred deaths in infant boys and girls annually are due to smoking during pregnancy (14). Worldwide prevalence of tobacco use is estimated to be about 29\% and it is still rising (15). Attractive tobacco promotion and the influence of the tobacco industry on policies have led to this continuous growth of smoking (4).

Nicotine addiction is one of the problems associated with cigarette smoking. Nicotine is one of the 4000 chemicals found in the smoke from cigarettes, cigars and pipes. Most US cigarettes contain 10 mg or more of nicotine (16). Nicotine is rapidly absorbed through the skin and mucus lining of the mouth and nose, or by inhalation in the lung. Once absorbed, nicotine rapidly distributes in the blood stream and reaches the brain within seconds (17). In the brain,
nicotine increases the level of dopamine, a brain chemical involved in mediating the desire to consume more nicotine, causing a smoker to continue smoking throughout the day to maintain pleasure effects and prevent withdrawal symptoms (18). This often leads to nicotine addiction/dependence and smokers continue to smoke despite negative health consequences (16). Among the 75%–85% of smokers who would like to stop, less than 50% of them succeed at permanent cessation (19). The rates of nicotine dependence are higher among females, whites (20) and middle-aged current smokers (21) when compared with males, minorities and older adults. In addition to this, a significant correlation exists between daily smoking and lifetime nicotine dependence to early age of smoking onset, low education, Hispanic ethnicity, parental and peer smoking, novelty seeking, and pleasurable initial smoking experiences (22).

Despite widespread knowledge of smoking-related illness, tobacco stores and hookah bars continue to operate. The main reason for an increase in hookah and cigar use is due to the misinformation about the product. It is oftentimes erroneously believed that smoking hookah or cigars involves less toxic chemicals and therefore is a safer alternative to cigarettes (23, 24). Electronic cigarettes (e-cig) are an emerging public health concern among the younger generation. Similar to the hookah, e-cig are marketed as a safer alternative for conventional cigarettes as no burning of tobacco is involved (25). However in 2008, WHO (World Health Organization) declared that e-cig cannot be used as a method to help quit smoking. Inadequate research demonstrating e-cig effectiveness and evaluation of the possible risks to human health derived from nicotine, flavorings and propylene glycol (the major ingredients of e-cig) were the main reasons behind this decision (26).

Secondhand smoke (SHS) generated during cigarette smoking is emerging as a potential environmental threat because it is an indoor air pollutant affecting the health of non-smokers (27). SHS, often termed environmental tobacco smoke (ETS), is another emerging public health problem for the non-smoker population. SHS mainly consists of the mixture of mainstream smoke (exhaled by a smoker) and side stream smoke (smoke from the lighted end of the cigarette)
(28). As the non-smoker breathes toxic chemicals similar to active smokers, it is also called involuntary smoking or passive smoking (29). People are more prone to SHS exposure at work, public places, home, and in the car (30). SHS contains about 7000 chemicals among which 70 are considered to be carcinogens (31). Therefore, exposure to SHS may increase the risk of nasopharyngeal and lung cancer as well as cardiovascular disease. SHS is more problematic in young children as it may trigger asthma attacks, ear and lung infections, and even sudden infant death syndrome (SIDS)(31). Recently, third hand smoke (THS) is also being considered a potential threat to non-smokers. THS mostly consists of residual tobacco smoke pollutants that settle on surfaces and react with other indoor pollutants to form a complex mix (32). This mix may pose a potential health hazard to non-smokers, especially young children (33).

The popularity of smoking decreased slowly around the late 20th century as people became aware of the health effects of both active and passive smoking. The public became aware about how the cigarette industry could mislead and influence public policy according to their interests (4). Public health activism led to the successful implementation of strict 100% smoke free workplace laws in 24 states and the District of Columbia, which helped to decrease the prevalence of smoking to almost half of what it was in the 1960’s (34). This contributed to a lower prevalence of cigarette consumption as smoke-free workplaces encouraged smokers to quit smoking (35).

The real public health problem is to understand and deal with tobacco-related illness. Solid evidence has been provided by several epidemiological studies linking smoking with cancer and heart disease (36, 37). Cigarette smoke is recognized as important risk factor for chronic obstructive pulmonary disease (COPD). It is the third most common cause of death in the US and more than 12 million Americans are known to have COPD (38). Approximately 50% of smokers will eventually develop COPD, so the longer a person smokes, the higher the risk for COPD (39). The number of deaths from COPD among ex-smokers is reported to be much lower than in current smokers (40). All these indicate the role of cigarette smoke in the progression of COPD.
According to WHO more than 3 million people died of COPD in 2005 and by 2030 it will become the third-leading cause of death worldwide (39). It also poses a huge economic burden of approximately $50 billion (including $20 billion in indirect costs and $30 billion in direct health care expenditures in 2010)(38). In addition, COPD results in chronic inflammation of airways and lung parenchyma, mucus hypersecretion, ciliary dysfunction, destruction of alveoli, emphysema and gas exchange abnormalities (41).

A report titled “Smoking and Health” released by the US Surgeon General reinforces the perception that cigarette smoke is harmful (14). The report highlighted the strong association of cigarette smoke with oral and laryngeal cancer and how smoking promotes the risk of heart disease (14). This report has influenced and convinced many smokers to quit (15). This report also helped to implement several public health interventions such as laws prohibiting the sale of tobacco to minors, restrictions in cigarette smoking, and increases in cigarette prices through imposition of taxes (42). The epidemic of smoking-related disease is the greatest public health threat of the twentieth century. Therefore, a decline in cigarette smoking and its consequent effect on the health of individuals would be surely one of the public health great successes (14).

1.3 History of alcohol

The Father of Toxicology, Paracelsus, coined the term “alcohol” to describe the product of wine distillation. The word alcohol is derived from the Arabic word “alkohol” meaning “bloom,” or the thinnest component of wine. Alcohol in its clear form without water was first prepared in 1796 through a special distillation process (43). In ancient times, alcoholic drink was made from the fermentation of berries, honey, grapes, corn or maize (44). Since its discovery, consumption of alcoholic beverages has become a part of human life, and an integral part of various religious ceremonies and social events. In the initial period after its discovery, alcohol was considered to be a medicine rather than a drink for enjoyment (45). Alcohol was extensively served as food, medicine, euphoriant and was expected to prolong life as well as revive and maintain the heart.
People enjoyed drinking on several occasions, especially during memorial ceremonies offering sacrifices to gods and ancestors, celebrating victories, births, marriages, reunions, and even deaths (45). Availability of various organic materials and the improvement of fermenting techniques has led to increased production of thousands of different alcohol beverages, both commercially and non-commercially, throughout the world (46).

1.4 Alcohol abuse and public health threat

During the second half of the 18th century, alcohol was considered an evil. Drinking alcohol was considered a sin and it was attributed to personal, economic, criminal, family and social problems. The consequences of alcohol abuse were thought to be severe and unacceptable to society (45). Today, alcohol is the most frequently abused drug worldwide (46). Alcohol abuse is one of the major public health problems. Alcohol is still the third leading cause of preventable death in the US resulting in 88,000 deaths annually. Alcohol is also a huge economic burden to the US economy. In the US in 2006, alcohol misuse problems cost $223.5 billion. Interestingly, three quarters of this total cost was due to binge drinking, while the remaining cost was due to underage drinking and drinking during pregnancy (47). In the US half of the general population regularly consumes alcohol (46) and approximately 17 million people are diagnosed with an alcohol abuse disorder that occurs when a drinking pattern causes both distress and harm. Unhealthy use of alcohol is highest among patients with trauma (50%), but also seen among patients in emergency departments (30-40%) and outpatients (1-20%) (48). Alcohol abuse results in increased disease and injury, property damage, motor vehicle crashes, alcohol-related crimes, and lost work productivity (49). Ethnic and culture disparity in alcohol-related problems is also a public health concern as alcohol-related mortality is higher among black than whites. In large urban hospitals, approximately 20-40% of patients suffer from illness either caused by alcohol or made worse by alcohol consumption (46, 50).
Misuse of alcohol is the main problem (44). When the pattern of drinking becomes destructive, then it could lead to alcohol abuse. The destructive pattern often termed as binge drinking happens after 4 or more drinks in women and 5 or more drinks in men within a 2 hr period time, respectively. This could result in a blood alcohol content (BAC) of 0.08% (17.6 mmol/L) and increases the risk of immediate adverse consequences (51). According to NIAAA (National Institute of Alcohol Abuse and Alcoholism), approximately 18 million adults have alcohol use disorders (AUD)(52). People with AUD are unable to control drinking and continue to use alcohol despite its negative effects resulting in alcohol dependence. They will show withdrawal symptoms when drinking is decreased or completely stopped (53). AUD is the most common psychiatric disorder in the general population; more common in men and often going undiagnosed (54). Alcohol abuse has several negative health consequences. In liver, heavy drinking could result in fatty liver, alcoholic hepatitis and cirrhosis. In brain, heavy drinking could have a wide range of effects. Heavy alcohol consumption can change the activity of neurotransmitters and cause an imbalance in neurotransmitters resulting in seizure, sedation, depression, agitation, mood and behavior disorders. This may also contribute to the “high” of intoxication and craving to drink during alcohol withdrawal (55). Also during chronic alcohol use, abrupt cessation unmask the adaptive response of the brain to alcohol resulting in withdrawal symptoms (56). Thus, people drink to experience a rewarding stimulus known as alcohol-induced euphoria, or to prevent/alleviate the anxiety they experience during alcohol withdrawal (53).

In spite of increased awareness among the public regarding the adverse consequences of alcohol abuse, thousands of people die from alcohol-related causes annually making it the third leading cause of preventable death in the US. Although the implementation of laws that regulate alcohol sale and distribution and penalize alcohol abuse-related activities, alcohol abuse is still common throughout the world (46).
1.5 Alcohol abuse and cigarette smoking

One of the important characteristics of alcohol abusers is that they also smoke cigarettes. Smoking is common among people who drink large amounts of alcohol. Approximately 90% of the people who abuse alcohol smoke cigarettes with 70% of them smoking at least one pack of cigarettes per day (58). Smoking is highly prevalent among heavy drinkers (59) and alcoholism is more common among smokers than non-smokers. The majority of alcoholic smokers have difficulty in quitting smoking when compared to non-alcoholic smokers (60). Drinkers are three times more likely to be smokers than non-drinkers and vice versa. Heavy drinkers smoke more than a pack of cigarettes a day (61). Increased alcohol use and binge drinking are associated with current smoking (35).

Alcohol dependence among smokers is common as drinking and smoking frequently occur together (62). In 2006, 46 million adults reported using alcohol and tobacco together and 6.2 million had AUD and nicotine dependence (63). The rate of smoking is high among alcohol abusers as approximately 90% of outpatient alcohol abusers smoked cigarettes (64). This suggests a high prevalence of smoking among alcohol abusers (59). Also a relationship exists between the amount of tobacco consumed and the severity of alcohol dependence. Current smokers and ex-smokers are more likely to consume alcohol than non-smokers (65). In AUD, heavier cigarette smoking is more common and persistent than in non-AUD. People who abuse alcohol are more likely to consume higher quantity of cigarettes and remain as a smoker for a long time (65). Similarly, severe alcohol dependence and consumption of alcohol in greater quantity and frequency occur in alcohol-dependent individuals who smoke compared to who those who don’t smoke. This results in significant health consequences among alcohol abusers who smoke. When these substances are used together, they exert synergistic effects that could potentially lead to increased risk for oral/pharyngeal cancer (66). This is greater than the sum of the independent risk of those substances (67). Certain cancers of the mouth, throat and esophagus are common in such people (68). In addition, people who smoke cigarettes and drink alcohol are known to increase
their risk of lung infection by various strains of bacterial, especially *Streptococcus pneumoniae* (69, 70).

Alcohol has multifactorial actions as it affects every organ and tissue in the body. Both oxidative and non-oxidative metabolism of alcohol plays an important role in tissue-specific damage (71). Interactions with several signaling proteins and ion channels important for cellular signaling are involved in alcohol-mediated injury. This results in disruption of lung defenses, cytokines and chemokine release, neutrophil chemotaxis, and production of reactive oxygen species (72). Oxidative stress, inflammation, acetaldehyde generation and adduct formation, decreased barrier function, and mitochondrial injury are some of the known pathological mechanisms identified as causative factors in alcohol-mediated tissue and organ injury (73). In the case of cigarette smoking, inflammation is the common feature for the pathogenesis of cigarette smoke-associated disease. The recruitment of inflammatory cells, such as monocytes and neutrophils, to the lung results in the release of toxic mediators, proteolytic enzymes and reactive oxygen species (74). This leads to damage of the extracellular matrix protein and tissue, which play a key role in the pathogenesis of tobacco-related disease (75, 76). In addition to tissue damage, inflammatory cells and smoke-mediated oxidative injury also participate in the pathogenesis of smoking-related illness (77).

Only a few pathologic mechanisms have been introduced regarding the injury associated with combined alcohol and cigarette smoke. As the airways are directly exposed to inhaled cigarette smoke and exhaled alcohol, the lung represents the ideal target for the effects of co-exposure. It is not clear how the co-exposure to smoke and alcohol play a role in lung disease even-though approximately 70% of alcoholics smoke cigarettes. It is known that alcoholics who smoke are at increased risk for developing lung infections (78). Alcohol and cigarette smoke results in reduced bacterial clearance due to reduced ciliary beat frequency (79). Co-exposure to alcohol and cigarette smoke results in increased colonization of *S. pneumoniae* in lung due to decreased mucociliary clearance (70). One important mechanism could be generation of reactive
aldehydes because they can be formed both from alcohol metabolism and pyrolysis of tobacco. Such aldehydes are accumulated in an increasing amount resulting in the formation of DNA and protein adducts by modifying target macromolecules. These modified macromolecules could be of clinical importance as they could play an important role in lung pathophysiology (79).

1.6 Significance and clinical relevance

During consumption of alcohol, about 95% of ingested alcohol is absorbed by the gut while the remaining 5% is eliminated in its original form via several excretory organs (kidney, skin and lungs). After absorption, 80% of alcohol is metabolized in the liver. The remaining 20% of alcohol travels to the lungs via the pulmonary circulation and diffuses from the bronchial circulation through epithelium (80). Once in the airways, alcohol vaporizes and tries to escape out in the exhaled breadth, but gets condensed back into the airways. Due to this recycling effect often called the “rain effect,” the airways are continually exposed to high concentrations of alcohol. Therefore, during chronic alcohol consumption or alcohol abuse conditions, the airways are bathed in high concentrations of alcohol for a prolonged period of time (81). In the airways, alcohol is further metabolized to acetaldehyde by the action of three main enzymes known as alcohol dehydrogenase, CYP2E1 and catalase (82).

In chronic alcohol consumption, lung alcohol is primarily metabolized through the CYP2E1 pathway. During this pathway, reactive oxygen species are produced which leads to lipid peroxidation generating another aldehyde, malondialdehyde (83). Because most of these alcohol abusers are heavy smokers, a higher concentration of these reactive aldehydes gets accumulated in the airways of the smoker-drinker as burning the cigarette itself generates high concentrations of acetaldehyde. In addition, reactive oxygen species contained in the smoke can lead to lipid peroxidation generating more malondialdehyde (84). Importantly, these aldehydes are highly reactive and electrophilic in nature and have the tendency to attack nucleophilic amine groups contained in proteins or nucleotides in DNA forming different stable and unstable adducts.
Depending upon their target, the adduct could be termed as DNA adduct or protein adduct (85). When there is a reaction of amino group with aldehydes, a new aldehyde-modified protein (adduct) is formed. These different types of adducts formed have been shown to have different physiological effects. DNA adducts have been shown to lead to damage in DNA, especially base pair and frame shift mutations (86). The hybrid protein MAA adduct is highly stable and immunogenic in nature (87).

Even though a few studies have been done to identify hybrid protein adduct formation in lung, no studies have been done to identify the DNA adduct formed in lung due to drinking and cigarette smoking. Most of the studies are focused on the individual effect of either cigarette smoke or alcohol exposure on lung function, but it is necessary to study the co-exposure affects on various lung adduct formation. Therefore, it would be important to identify the aldehyde DNA adduct formed in the lung due to smoking and drinking (84) and understand its lung effects. The hybrid adduct has been shown to play an important role in inflammation (88) and is primarily taken up by scavenger receptor A (SRA; CD204) predominantly expressed in macrophages. SRA is one of the major receptors known to bind aldehyde-modified proteins. In spite of this, no study has yet been done to understand the effects of hybrid adduct in macrophage functions and the role of scavenger receptor in mediating such effects. It is also necessary to identify the role of SRA in aldehyde adduct-induced lung inflammation. Therefore, it is important to identify different aldehyde adducts formed in lungs of smokers and drinkers and understand how their formation and accumulation in lung affects lung physiologic functions.

As exhaled alcohol and inhaled cigarette smoke is being continually exposed to airways, the lung represents an important target for the effects this co-exposure. Because such a high percentage of alcoholics smoke cigarettes, it is necessary to further study how these reactive aldehydes generated in the lung in such a high concentration could play a role in pathogenesis of lung diseases. Herein, we hypothesize that aldehyde adducts formed in the lung after co-exposure to alcohol and cigarette smoke results in lung injury. Our study will help to develop novel
pathway targets that might prevent or treat adverse health effects of such exposure. Our study will help to identify these aldehydes and their adducts and understand their role in progression of lung disease in drinkers who also smoke.
CHAPTER TWO:

2. Alcohol, Aldehydes, Adducts and Airways

2.1 Introduction

Lung

The lung is a highly specialized organ charged with the principal role of $O_2/CO_2$ exchange between atmosphere and bloodstream (89). In addition to this gas exchange, it also serves as an interface between host and external environment (89). In this regard, the lungs can be considered an external organ due to continual exposure to ambient air (90). The enormous surface area of the airways and continuous exposure to external air makes the lung vulnerable to numerous inhaled toxicants, gases, pathogens and chemicals (90). All of these exposures make the lung susceptible to varying degrees of physical, chemical, and biological insults (91). To combat these insults and to defend against inhaled pathogens and other toxicants, the lung employs a defense mechanism including exhalation, cough reflex, ciliary beat, and mucus clearance (92), as well as a highly complex innate immune system including airway epithelial cells (93) and resident and recruited leukocytes (90). This first line of defense is later followed by specific acquired immune responses associated with the activation of T and B cells aimed against specific antigens (94). In the case of continuous insults, lung defense is compromised; allowing inhaled toxic agents to stimulate the generation of reactive oxygen species (ROS) (95). These ROS induce intracellular responses resulting in the release of pro-inflammatory cytokines and chemokines (95) that stimulate the influx of neutrophils and monocytes into the lung (95). Continuous inhalation of pathogens or toxic agents, however, may result in excessive ROS production, leading to chronic inflammation and lung injury (96). If not controlled, these ROS may induce inflammation and DNA damage, inhibit apoptosis, and may also activate proto-oncogenes through initiation of several signal transduction pathways (97). Therefore, oxidative stress associated with increased production of
ROS in the lung due to various toxic inhalants may predispose individuals to lung diseases such as chronic obstructive pulmonary disease (COPD)(98).

2.2 Role of alcohol in lung disease

Chronic alcohol abuse or alcoholism costs about $223 billion annually to the U.S. economy (49) and is the fourth leading preventable cause of death, causing more than 88,000 deaths annually (99). Drinking more than two drinks/day for men and one drink/day for women can have deleterious health effects and is associated with increased mortality (100). Among critically ill patients alcohol abuse is common and attributed to about 40% of admissions to the intensive care unit (101). Tissue injury to liver, stomach and brain as well as cancers of the upper aero-digestive tract, stomach, and liver are known health risks associated with chronic alcohol consumption (102). In the lung, alcohol over-consumption predisposes the host to infectious diseases such as pneumonia (103) as well as acute respiratory distress syndrome (ARDS)(104). After oral ingestion, less than 10% of alcohol consumed is excreted unchanged in urine, sweat and breath (105, 106). In the lung, due to alcohol’s volatility, it diffuses freely from the bronchial circulation into the airways, rapidly condenses with decreasing temperature, and deposits back onto the airways. This recycling of alcohol vapor (or “rain effect”) potentially results in a high concentration of alcohol in the airways (81). Thus, exhaled alcohol breath tests are commonly used to measure alcohol ingestion by law enforcement agencies in estimating blood alcohol levels (107).

Bacterial infection and acute lung injury are the most significant pulmonary effects of such alcohol abuse (108). Increased risk for infection with tissue-damaging gram-negative pathogens, such as *Klebsiella pneumonia*, is common in alcoholic patients (109). Other risks associated with alcohol abuse are aspiration of gastric acid and/or microbes and impairment of mucous-facilitated clearance of bacterial pathogens (108). In part, this explains the increased risk of respiratory infections in individuals with alcohol use disorders (AUDs). Alcohol-mediated
suppression of host immune response and pathogen-clearing function of alveolar macrophages could further explain the increased risk of both bacterial pneumonia and tuberculosis (110).

Production of white blood cells in the bone marrow and superoxide production in neutrophils are also decreased in chronic alcohol consumption (111). Chronic alcohol consumption increases alveolar capillary permeability, protein concentration in the alveolar lining fluid and pulmonary edema formation in lung (112). Chronic alcohol ingestion also depletes the antioxidant, glutathione (GSH), throughout the alveolar lining fluid of the lung and within macrophages (104). Other deleterious effects include abnormal synthesis and secretion of lung surfactants and increased apoptosis of type II cells (113).

Although alcohol has many adverse effects on lung function, only a limited number of studies have examined the biochemical processes involved in the mechanism of such injury. Interaction of alcohol’s metabolites with other exposures could be one of several possible causes (114). Therefore, development of alcohol co-exposure markers in the lung could be of interest in understanding the pathogenesis of lung disorders associated with alcohol abuse.

2.3 Role of cigarette smoking in lung disease

Cigarette smoking is the number one preventable cause of death in the United States, resulting in 480,000 deaths each year (115). A causal association between cigarette smoking and cancers of lung, liver, nasopharynx, oropharynx, and larynx has been established by epidemiological studies (68, 116, 117). In developed countries, cigarette smoking attributes to approximately 90% of lung cancer cases in males and 80% in females (118, 119). The number of cigarettes smoked, inhalation practice, duration and early start of smoking are the critical risk factors (120). Thousands of chemicals contained in tobacco smoke are known to have carcinogenic properties and can undergo metabolic activation in tissue leading to formation of reactive intermediates (121, 122). Besides being a risk factor for cancer development, smoking is also the main cause for COPD development (123).
Cigarette smoke contains high concentrations of free radicals in both the gas and tar phases (120). These stable oxidized intermediates induce endogenous oxidative stress and inflammation (124). Oxidative DNA damage and lipid peroxidation (LPO) of cell membranes are important effects of cigarette smoke-induced oxidative injury (125). LPO provides a continuous supply of free radicals for the oxidation of polyunsaturated fatty acids in membranes causing oxidative cell damage (126). Cigarette smoke-mediated oxidative stress induces local inflammation resulting in increased numbers of macrophages in the lung (127). These macrophages recruit additional inflammatory cells into the lung including neutrophils, monocytes, eosinophils, and T lymphocytes (128). The result is a destructive cascade of exposure of the elastolytic compounds and ROS that destroy the lung structure resulting in emphysema and obstructive bronchitis (129). In addition, high carbonyls content such as acrolein and 4-hydroxynonenal (4-HNE) in the cigarette smoke also leads to carbonyl stress in the lung (130). Other carbonyl compounds present in cigarette smoke are formaldehyde, acetaldehyde, propanal and malondialdehyde (131). These carbonyls generated as a result of oxidative stress may play an important role in the progression of lung disease such as COPD (132).

2.4 Other environmental oxidants

In recent years, ambient air pollutants and diesel exhaust particles have been linked to oxidative damage in cells and in tissue (133). Air pollutants also contribute to oxidative stress in the pulmonary system and play a role in adverse lung effects (134). Ozone, a secondary air pollutant, is a known pulmonary irritant (135). In addition to a single agent, exposure to combined air pollutants, such as ozone and particulate matter (PM), greatly induces pulmonary oxidative stress and inflammation (135). This could explain the association between environmental air pollutants and increase in pulmonary diseases and mortality demonstrated by several clinical and epidemiological studies (136-138).
2.5 Source of aldehydes in the lung

Significant amounts of ingested alcohol reach the airways via the bronchial circulation where it is either metabolized or is excreted by exhaling the vapor (82). Although the majority of ingested alcohol is metabolized in the liver, the mammalian lung can also metabolize ingested alcohol through the action of alcohol dehydrogenase (ADH) to acetaldehyde (82). Thus, after alcohol consumption, airways are exposed to high concentrations of acetaldehyde, a primary metabolite (139). In addition to ADH, during chronic alcohol consumption, alcohol is metabolized by microsomal cytochrome P450 2E1 (CYP2E1) and peroxisomes to generate ROS leading to oxidative stress (140). Human lung cells, especially bronchial epithelium, club cells, type II pneumocytes, and alveolar macrophages, have been shown to express CYP enzymes (141). CYP2E1-generated ROS easily react with lipid membranes causing LPO (142), which is important in the generation of reactive aldehydes such as malondialdehyde (MDA) and other products, like 4-HNE (143, 144). 4-HNE forms Michael adducts with nucleophilic sites in DNA, lipids and proteins (144). Another major source of reactive aldehydes in the lung is from the vapor phase of cigarette smoke, which is known to contain several aldehydes including butyraldehyde, isobutyraldehyde, propionaldehyde, and acetaldehyde (145)(Fig. 1). Among the different aldehydes contained in cigarette smoke, acetaldehyde is the major one, presenting in very high concentrations (146)(approximately 920 µg per cigarette)(147). Additionally, acetaldehyde is widely used as a natural constituent of foods and is present in the environment as a pyrolysis product (148). Acetaldehyde and MDA also are produced in biologically significant amounts during the metabolism of alcohol (149). Higher levels of aldehydes have also been reported in exhaled breath condensate and saliva in current smokers and patients with COPD (150, 151). Aldehydes have also been identified in the bronchoalveolar lavage (BAL) fluid of animals exposed to ozone (152). These aldehydes, especially acrolein, MDA, formaldehyde and crotonaldehyde, are highly reactive and could form DNA adducts in a variety of human tissues (153)(Table. 1). Additionally, significantly elevated levels of DNA adducts and smoking-related
protein adducts were detected in BAL cells as well as in the bronchial epithelium and the peripheral lung of smokers (154, 155). Lung is also vulnerable to oxidative injury as a result of exercise and high altitude exposure due to oxidative stress (156). In addition to the lung, increased MDA levels are also reported in excreted urine of patients with COPD after exercise as a result of exercise-induced stress (157).

2.6 Pathological implications of lung aldehydes

The accumulation of LPO products in human tissues is a major cause of cellular and tissue dysfunction as it may lead to membrane dysfunction and oxidative stress-related diseases (158). Reactive α, β-unsaturated aldehydes generated as a result of LPO could contribute to vascular disease and other oxidative stress-related pathologies due to modification of biomolecules (159). Because oxidative stress plays an important role in the development and/or progression of vascular diseases such as atherosclerosis, serum malondialdehyde & malondialdehyde-acetaldehyde levels are used as biological markers of oxidative stress (160). 4-HNE, a highly reactive end product of LPO (98) has been linked to a number of pathologies such as alcoholic liver disease, COPD, emphysema, asthma, Alzheimer’s disease and Parkinson’s disease (161). In the lung during oxidative stress, reactive LPO products are degraded very slowly, resulting in greater accumulation of these products leading to extensive adduct formation and tissue damage (144). Acetaldehyde may also trigger asthma attacks in individuals with genetic alcohol dehydrogenase polymorphisms (162). Inhalation of acetaldehyde for 30 minutes causes mild respiratory irritation (163). Acetaldehyde also has a significant role in the etiology of lung cancer (143). Aldehydes in cigarette smoke are able to induce the pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) from macrophages, and the neutrophil chemokine, interleukin-8 (IL-8), from human bronchial epithelial cells (164, 165). Aldehydes contained in cigarette smoke have been shown to inhibit human neutrophil apoptosis and contribute to neutrophilic accumulation, resulting in the delayed resolution of inflammation.
Acrolein, one of the major constituents of cigarette smoke, is involved in increased mucin production and regulation of lung matrix metalloproteinase 9 (MMP-9), which may result in decreased lung function in COPD patients (167). In addition to this, glutathione is irreversibly modified by acrolein and crotonaldehyde in human airway epithelial cells (168). Acetaldehyde is also considered a toxin with epigenetic and genetic effects (169). Ethanol-induced hepatic steatosis, fibrosis, carcinoma and gastrointestinal injury are attributed to alcohol-mediated oxidative stress (170). Lipid peroxidation affects mitochondrial membrane permeability (171). Similarly, acetaldehyde could also inhibit mitochondrial reactions and functions (171). Acrolein may have a role in epigenetic modification as it is known to form adducts with histone protein (172). In brief, in addition to oxidative stress and immune dysfunction, membrane disruption, histone modification and mitochondrial dysfunction are other major pathological implications of aldehydes.

Even though chronic alcohol ingestion and cigarette smoke are two major sources of aldehydes in the lung, few studies have been conducted on the co-exposure of alcohol and cigarette smoke in the lung. This co-exposure is important because the highest level of aldehydes is generated when lungs are co-exposed to cigarette smoke and alcohol (84). This co-exposure often leads to oxidative stress resulting in high concentrations of acetaldehyde and malondialdehyde in the lung (84). Therefore, the reactive aldehydes generated in the lung could be related to various lung pathologies associated with alcohol abuse and cigarette smoking.

2.7 Lung aldehydes and protein adduction

Reactive aldehydes are electrophilic and react with a nucleophilic site that donates an electron to form a strong covalent bond leading to adduct formation (173)(Fig. 1). Generally, one of two chemical reactions are involved in adduct formation (174). One is a Michael addition, which is the reaction between β-carbon of aldehydes and nucleophilic groups to form 1,2-addition with the double bond. Secondly, there is a base reaction that involves formation of Schiff bases between
the carbonyl carbon of aldehyde and the primary amino group of lysine or N-terminal residues (175). Various stable and unstable adducts are formed when reactive aldehydes generated covalently bind to amino acid residues of proteins (176). Such adduction may disturb protein cellular functions (177)(Table. 1). Aldehydes damage protein structure by forming adducts through covalent bonding with cysteine, lysine or histidine residues (178). In the lung, both ADH- and CYP2E1-catalyzed metabolism of alcohol is associated with generation of acetaldehyde, a reactive aldehyde capable of binding to cellular proteins (142). Acetaldehyde, the first metabolite of alcohol, is highly reactive and forms adducts primarily by binding to reactive lysine residues of preferred target proteins (179). Acetaldehyde also has the ability to covalently bind to several proteins that could be detrimental to the protein function (180) due to formation of both stable and unstable adducts with various proteins (181). In addition, other lipid peroxidation-generated aldehydic products such as MDA and HNE also form Schiff base adducts with proteins (182).

Several proteins such as albumin, tubulin, lipoproteins, collagens and erythrocyte membrane proteins serve as targets for aldehyde adduction (183). Accumulation of acetaldehyde due to excess alcohol consumption can lead to increased interaction of this aldehyde with biomolecules (114). Tobacco smoke is another source of oxidative stress in the lung as it induces the production of aldehyde-mediated injury through oxidative DNA damage and lipid peroxidation of cell membranes (184). In addition to acetaldehyde, 4-HNE has been known to form protein adducts with insulin and histidine residues in proteins (185).
1. \( \text{CH}_3\text{CH}_2\text{OH} \xrightarrow{\text{ADH}} \text{CH}_3\text{CHO} \)
   - Alcohol
   - Acetaldehyde
   - $\text{NAD}^+$
   - $\text{NADH}$

2. \( \text{CH}_3\text{CH}_2\text{OH} \xrightarrow{\text{CYP2E1}} \text{CH}_2(\text{CHO})_2 + \text{C}_6\text{H}_5\text{O}_2 + \text{CH}_2=\text{CHCHO} + \text{HCOH} \)
   - Alcohol
   - Malondialdehyde
   - 4-Hydroxynonenal
   - Superoxide $\text{O}_2^-$
   - Hydroxyl radical OH
   - Hydrogen peroxide $\text{H}_2\text{O}_2$

3. Cigarette smoke
   - $\text{CH}_3\text{CHO} + \text{C}_6\text{H}_5\text{O}_2 + \text{CH}_2=\text{CHCHO} + \text{HCOH}$
   - Acrolein
   - Formaldehyde
Figure 1. Generation of lung aldehydes and adduct formation.

Alcohol is metabolized by alcohol dehydrogenase (ADH) to acetaldehyde (AA). But during chronic alcohol consumption, CYP2E1 is induced leading to generation of ROS like superoxide, hydrogen radical and hydrogen peroxide. This promotes lipid peroxidation and generation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Cigarette smoke itself contains high concentration of AA, acrolein and formaldehyde. In addition to this, smoking cigarettes also induces local inflammation in lung causing more generation of ROS. This further promotes lipid peroxidation generating more MDA and 4-HNE. Acetaldehyde and MDA could form hybrid adduct through Schiff base reaction when 2 mole of MDA react with 1 mole of AA to form a stable hybrid adduct. Aldehydes like AA, MDA, acrolein, 4-HNE and formaldehyde could also form protein adduct and DNA adduct. The main reactions involved are Schiff base (involves binding of aldehyde to the alpha group of a N-terminal amino acid of the protein) and Michael addition (involves of binding of aldehyde on amino groups (Lys and His) or thiols (Cys or GSH)(88, 186-188).
2.8 Pathological implications of protein adducts

Slow cilia beating and decreases in cilia dynein ATPase activity have been reported as a result of acetaldehyde binding with dynein and tubulin proteins important for cilia motion (189)(Table. 1). Adduction of acetaldehyde with GSH inhibits the anti-oxidative defense system (AODS) responsible for the detoxification of ROS and reactive nitrogen species (RNS)(190). Stimulation of nuclear factor-kappa B (NFκB), which regulates the secretion of pro-inflammatory cytokines, is another effect of acetaldehyde (191). Aldehyde products stimulates fibro genesis by increasing the expression of connective tissue proteins and extracellular matrix components (192, 193) and induces immune responses (194-196). Also, 4-HNE formed during lipid peroxidation after ozone exposure appears to form specific protein adducts which is toxic and cause apoptosis of murine lung cells (197). Acute alcohol toxicity may lead to formation of malondialdehyde protein adduct in the muscle (198). The presence of HNE-protein adducts has also been studied in diseases related to oxidative stress such as neurodegenerative diseases and atherosclerosis (199). Similarly, protein adducts of acrolein may have a role Alzheimer's disease, Parkinson's disease (200) atherosclerosis (201) and chronic obstructive lung disease (202). Exposure of 4-HNE to THP-1 cells resulted in modification of proteins and enzymes involved in cytoskeleton organization, stress responses, and other metabolic pathways (203). MDA and 4-HNE protein adduct formation in the liver could play an important role in the development and progression of alcoholic liver disease (204, 205). In COPD patients, a large number of carbonyl-modified proteins has been reported in the peripheral lung tissue and correlated with disease severity measured by the decline in forced expiratory volume in 1 second (FEV1)(206). Aldehyde-modified protein formation also has an effect on cellular responses. 4-HNE adduction with extracellular signal-regulated kinases (ERK1/2) decreased ERK-1/2 phosphorylation and nuclear localization (207). Similarly, the modification of adenosine monophosphate-activated protein (AMP) kinase with 4-HNE inhibits its kinase activity and attenuates downstream AMP kinase signaling pathway in MCF-7 breast cancer cells (208). HNE forms adducts with c-Jun amino-
terminal kinases (JNKs) leading to nuclear translocation and activation in human hepatic stellate
of extracellular matrix protein, which could lead to the formation of scar tissue in the liver (149).
MDA, and 4-HNE modified proteins has also been studied in human eye disease (209) and
human hepatic stellate cells (210). Aldehyde adduct also interferes with the function and may
damage such macromolecules (124).

2.9 Lung aldehydes and DNA adduction
Acetaldehyde is highly reactive and the electrophilic nature of its carbonyl carbon results in
reactions with DNA, generating DNA adducts (211)(Fig. 1). This could explain the cytotoxic,
genotoxic, mutagenic, and clastogenic nature of acetaldehyde as DNA adduct formation plays a
critical role crotonaldehyde-derived propano-dG (CrPdGs) adduct (211). MDA is a natural
product of lipid in carcinogenesis (212, 213). Most of these effects have been proposed to
originate from a variety of DNA–acetaldehyde adducts (212). DNA base deoxyguanosine (dG) is
the major target for adduction followed by deoxyadenosine (dA) and then deoxycytosine
(dC)(214). Acetaldehyde forms other DNA adducts such as N2-ethyl-2′-deoxyguanosine (N2-Et-
dG)(215) and 1,N2-propano-2′-deoxyguanosine (PDG)(216). Another well-studied aldehyde-
DNA adduct is the peroxidation, is also capable of forming an exocyclic DNA adduct named
malondialdehyde-deoxyguanosine adduct (M1dG) after its interaction with DNA (217). MDA-
DNA adduct is also Malondialdehyde formed in the lung of cigarette smokers could form adducts
with DNA bases.

2.10 Pathological implications of DNA adducts
DNA damage is one of the important pathological conditions associated with DNA adduct
formation as this could increase the risk of somatic mutations (218) by inducing base pair
mutations and causing frame-shift mutations (219, 220)(Table. 1). MDA–DNA adducts in a
number of tissues, including liver (221) breast (222) and oral mucosal cells (223). Another DNA
adduct, M1dG, may be associated with increased cancer risk and tumor progression (218). MDA-DNA adducts might contribute to the cause of tobacco-related laryngeal cancer as these adducts have been detected in the bronchial epithelium and in the larynx of smokers (124, 222). Also, an increased level of MDA-dA was reported in the larynx of subjects with the highest intake of alcohol (>44 g)(224). M1dG adduct was also detected both in human bronchial epithelial cells and mouse lung tissue exposed to alcohol (225). In addition to lung, MDA–DNA adducts were also detected in tissues from patients with breast cancer (222). A correlation was found between CYP2E1, 4-HNE and exocyclic ethanol adducts of adenine and cytosine in patients with alcoholic liver disease (226). M1dG adducts were also detected on leukocytes exposed to formaldehyde (135) and industrial air pollution (139). DNA adducts formed by acetaldehydes could prompt replication errors and mutations in oncogenes or onco-suppressor genes, which increases risk for carcinogenesis (227).

2.11 Lung aldehydes and hybrid adducts

People with AUDs are two to three times more likely to smoke cigarettes than those without AUDs (58). This suggests more frequent and higher rates of cigarette smoking among those with AUDs than in the general population (228). Also a strong correlation exists between alcohol and tobacco consumption and heavy drinkers have more trouble quitting smoking than do light drinkers (229). In the lung, a unique aldehyde environment is created during co-exposure to alcohol and cigarette smoke due to the generation of a high concentration of aldehydes (84). For instance, high concentrations of acetaldehyde and malondialdehyde were detected in the BAL fluid of mice co-exposed to cigarette smoke and alcohol (84). This elevated level of aldehydes is of importance as it is necessary for the formation of the hybrid malondialdehyde-acetaldehyde (MAA) adduct in mouse lung (84)(Fig. 1). Formation of five different types of protein adducts, acetaldehyde, MDA, MAA, 4-HNE and hydroxyl ethyl radical, are reported to form after ethanol consumption (230). MAA-adducted proteins are highly stable and resistant to rapid degradation.
This hybrid adduct is composed of a cyclic product consisting of two molecules of MDA and one molecule of acetaldehyde as a result of a Schiff base reaction described previously (231). The MAA adduct is highly fluorescent and can be detected for a few weeks in the liver as a result of slow degradation (232, 233). In comparison to single exposure to alcohol or smoke alone, MAA adducts have been detected only in the lungs of mice exposed to both alcohol and cigarette smoke (84). Different endogenously nucleophilic proteins contained in the lung are the target of MAA to form hybrid adducts (84). Among these, surfactant protein A (SP-A) and surfactant protein D (SP-D) synthesized primarily by type II alveolar cell in the alveolus have been extensively studied (88). SP-A and SP-D play an important role in innate immunity as they can directly kill bacteria, or can act as an opsonizing agent by binding to bacteria subsequently enhancing macrophage phagocytosis (234). MAA-adducted proteins are good ligands for scavenger receptor A (SRA; CD204), which are expressed extensively on macrophages and also found on endothelial cells, platelets, and epithelial cells (235, 236). MAA stimulates inflammatory responses in airway epithelial cells through binding to SRA (237). Diminished antibody responses to MAA-bovine serum albumin (MAA-Alb) in SRA knockout mice have also been previously reported (235). In addition, pre-treatment with SRA-binding ligand, fucoidan, blocked MAA adduct-mediated release of pro-inflammatory chemokine IL-8 (238).
Table 1. Lung aldehydes, type of adducts formed and lung effect

<table>
<thead>
<tr>
<th>Lung Aldehydes</th>
<th>Sources</th>
<th>Lung Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde</td>
<td>Alcohol, Cigarette smoke, Environmental toxicants</td>
<td>Oxidative stress (159) COPD, asthma, emphysema (81, 106) Mild respiratory irritation (163) Release of pro-inflammatory cytokine (164) Epigenetic and genetic effect (170)</td>
</tr>
<tr>
<td>4-Hydroxynonenal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrolein</td>
<td></td>
<td></td>
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<tr>
<td>Formaldehyde</td>
<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lung Adducts</th>
<th>Aldehydes Involved</th>
<th>Lung Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein adduct</td>
<td>Acetaldehyde, Malondialdehyde, 4-hydroxynonenal</td>
<td>Damage protein structure and function (195, 209) Slow cilia beating (189) Inhibition of anti-oxidative defense (190) Stimulation of fibrogenesis (192, 193) and induction of immune response (195)</td>
</tr>
<tr>
<td>DNA adduct</td>
<td>Acetaldehyde, Malondialdehyde, Formaldehyde</td>
<td>Base pair mutation (219) Carcinogenesis (212, 213) Increased risk of mutation (218)</td>
</tr>
<tr>
<td>Hybrid adduct</td>
<td>Acetaldehyde, Malondialdehyde</td>
<td>Induce pro-inflammatory chemokine (238) Inhibit bronchial epithelial cell wound closure (239) Increase influx of neutrophils (88)</td>
</tr>
</tbody>
</table>
2.12 Scavenger receptor A

Scavenger receptor A (SRA) is one of the eight classes of scavenger receptor predominately expressed on macrophages and mast cells but are also expressed on dendritic cells, epithelial cells and endothelial cells (240). Scavenger receptor was first discovered as acetylated LDL receptor and was studied extensively for a long time in atherosclerosis for its role in uptake of low-density lipoprotein (LDL) (236). It is comprised of three related genes that encode at least five polypeptides, termed SR-AI, SR-AII, SR-AIII, macrophage receptor with collagenous structure (MARCO) and scavenger receptor with C-type lectin (SRCL) (241). The class A scavenger receptors are homo-trimeric membrane glycoprotein and consist of five different domains named relatively short N-terminal cytoplasmic domain, a single trans-membrane domain, the extracellular domain comprising of α-helical coiled-coils, multiple collagen-like repeats and a cysteine-rich C-terminal region respectively (242) (Fig. 2).

Figure 2. Structure of scavenger receptor A.
For ligand recognition, positively charged residues within the collagen-like repeats appears to be important. Once the ligand binds to SRA at the cell surface, it is delivered to the endosomes in clathrin-coated pits and vesicles. In the endosomes, due to the acidic pH environment, the ligands is released from the receptor and the receptor is subsequently recycled to the plasma membrane via the trans-Golgi network SRA (236).

Lately the role of the SRA has been widely explored due to its ability to bind to diverse ligands. Scavenger receptors can bind modified proteins, polynucleotides; pathogen associated molecular patterns like lipopolysaccharide (LPS), lipotechoic acid, maleylated bovine serum albumin, certain polyribonucleotides, some polysaccharides and even environmental particles like silica and asbestos polyvinyl sulfate (243, 244).

Scavenger receptor A was first discovered as its role in atherosclerotic plaque formation. SRA endocytose modified lipoproteins leading to formation of large lipid laden foamy macrophages especially found in such lesions. It also plays an important role in macrophage adhesion to the substrate and other cell populations. This is important for retention of these cells in the site of infections or inflammation. SRA also acts as a possible pattern recognition receptor and helps in binding and ingestion of microbes for anti-microbial defense. So it plays an important role in innate immunity. SRA also helps in clearance of apoptotic or unwanted host cells (Fig. 3). This would help to maintain normal tissue homeostasis (236, 245, 246).
Pathological implications of hybrid adduct

The hybrid adduct, MAA, has been reported to induce pro-inflammatory responses and delay wound healing in airway epithelial cells (Table. 1). MAA adduct stimulates release of the neutrophil chemokine, IL-8, when exposed to bronchial epithelial cells (238). Similarly, intranasal instillation of SPD-MAA in mice induced KC (CXCL1), a homolog of human IL-8, in comparison to saline or non-adducted SPD control (88). This elevation in KC release resulted in an influx of neutrophils in the lungs of mice instilled with MAA adduct for 3 weeks (88). MAA adduct-stimulated cytokine release is blocked by protein kinase C (PKC) inhibitors, implicating a role for PKC in MAA-adducted protein-stimulated IL-8 release from bronchial epithelial cells (238). MAA adducts have also been shown to inhibit bronchial epithelial cell wound closure (239). MAA adduct-induced inflammation is also mediated through PKC as MAA adducts activate PKC epsilon in tracheal epithelial cells (88, 238). Immunologic reactions associated with
alcohol-related liver disease and atherosclerosis-induced vascular inflammatory injury also have been associated with MAA adduct formation (247, 248). In addition to IL-8, MAA adducts have been reported to induce the expression of inflammatory cytokines such as TNF, intracellular adhesion molecule and vascular cell adhesion molecule in liver endothelial cells (249). Increased formation of MAA adduct has also been reported in rheumatoid arthritis synovial tissue (250). MAA adducts also induce an antibody response as T helper and cytotoxic T cells exhibit robust antibody responses to MAA epitope (251). Extent of tissue damage in acute injury and chronic disease states such as atherosclerosis could be correlated to this antibody response (248). Circulating MAA-modified proteins in the bloodstream could be bound, internalized, degraded and presented to the cells of the immune system resulting in an immune response (252). Formation of MAA adducts with N-terminal and bait region of mouse alpha 2 macroglobulin (A2M) has been shown to modulate its proteinase and TGF-b1 binding function (253).

2.14 Conclusion

Chronic alcohol consumption and cigarette smoking result in the production of several types of aldehyde adducts in the lung. The formation of these adducts leads to impaired function and induces inflammation and mutagenesis. Although chronic alcohol abuse predisposes the host to pneumonia and ARDS, very few studies have focused on the role of alcohol metabolism in alcohol-induced toxicity and its consequences in the lung. Many studies have been directed toward cigarette smoke-induced oxidative stress, but it has been shown that alcohol also increases LPO leading to the generation of reactive aldehydes such as acetaldehyde and MDA. Because the lung is continuously exposed to high concentration of alcohol in heavy drinkers, alcohol significantly contributes to the high level of aldehydes detected in the lung. Several mechanisms have been proposed to understand the consequences of alcohol–induced liver injury, but only limited studies have been done in the case of the lung. Additional studies are required to further clarify the role of alcohol in oxidative stress and aldehyde generation in the lung. Additional
studies are needed to determine the role of different adducts formed in the lung and their role in lung pathogenesis. As ROS-mediated lipid peroxidation is a major source of aldehyde generation in lung, it is also important to study different factors that stimulate ROS generation. Different reactive aldehydes and adducts formed in the lung could act as potential biological markers for the source and degree of lung injury associated with alcohol, cigarette smoke and other inhaled environment pollutants. Discovering innovative approaches to better identify the mechanisms through which adducts cause lung injury, however, still remains a challenge for researchers. Among all adducts, the stability of the MAA hybrid adduct may play a prominent role in mediating the long term consequences of chronic alcohol abuse and cigarette smoking with respect to the development of respiratory infections as well as emphysema and COPD. Understanding factors regulating adduct production and their role in the progression of chronic lung diseases is necessary and important in order to develop new therapeutic approaches targeting the formation and accumulation of reactive aldehyde adducts for promoting the resolution of lung injury.
CHAPTER THREE:
Protective Role of CYP 2E1 Inhibitor Diallyl Disulfide (DADS) on Alcohol Induced Malondialdehyde-Deoxyguanosine (M1dG) Adduct Formation

3.1 Introduction
The lung is exposed on a daily basis to a wide range of oxidants ranging from smog, diesel exhaust and dust particles to cigarette smoke and alcohol (254). Alcohol consumption in humans is a serious health hazard that may alter normal function and composition of lipid membranes (255) and is often associated with the increased occurrence of lung diseases like pneumonia and acute respiratory distress syndrome (151) and an increased cancer risk, suggesting a role for ethanol and its oxidative metabolites, acetaldehyde and malondialdehyde (MDA)(239, 256). People who are alcohol dependent are more likely than non-drinkers to be smokers (59), but alcoholics are also statistically more likely to die from smoking related diseases as opposed to alcohol-based illness (257). Cigarette smokers inhale thousands of carcinogens and free radicals (258, 259) that attach to the unsaturated fatty acid on cell membranes. This attachment results in a chain reaction that generates lipid hydroperoxides that decompose to MDA (260). MDA is genotoxic and forms DNA adducts that are involved in tobacco and alcohol-related cancers and cardiovascular disease (261).

Alcohol metabolism could be one of the important risk factor associated alcohol-related carcinogenicity (262). In the pulmonary epithelium lining environment (ELE) alcohol may act as a solvent and enhance the penetration of tobacco-related carcinogenic compounds (263). During alcohol ingestion there is a “recycling” effect where alcohol gets vaporized as it moves into the conducting airways and can deposit back into the airway lining fluid to be released again into the airways during exhalation leading to repeated exposure of the airway epithelium to high local concentrations of alcohol (81).
These compounds produce reactive oxygen species (ROS), which can attack DNA causing oxidized bases, and depurinating DNA adducts, leading to single and double DNA strand breaks (261, 264). Preventing or reducing the formation of DNA adducts is essential because chemical carcinogenesis is a multistage process involving initiation, promotion, and progression. Initiation requires the binding of a chemical carcinogen to DNA, thereby forming DNA adducts (265). DNA adduct formation is an important early step in carcinogenesis as endogenous DNA adducts derived from oxidative stress, lipid peroxidation, or other endogenous processes have been proposed as contributors to the etiology of human cancer (266, 267). Depurinating adducts such as MDA are electrophilic and preferentially attack one of the most nucleophilic positions on DNA; the N7 position of Guanosine (268). Specifically, the by-product of the reaction of MDA DNA is the pyrimido[1,2-a]purin-10-(3H) one nucleoside (M1dG) derived from deoxyguanosine, which has been shown to be mutagenic in bacterial and mammalian cells (269, 270). Apurinic DNA sites caused by M1dG can lead to mispairing when replicated in vitro by multiple different DNA polymerases (271, 272).

Diallyl disulfide (DADS) is an oil-soluble organosulfur compound derived from garlic known to be beneficially effective for human health (273) against carcinogenesis and chemically induced toxicity (274). This nutritional agent has also been shown to inhibit CYP2E1 metabolism (275, 276). Studies show an association of CYP2E1-mediated alcohol metabolism and adduct formation in the liver (277), juxtaposed with pulmonary CYP2E1 induction by carcinogens from pyrolyzed tobacco (278, 279). CYP2E1 plays a significant role in ethanol and cigarette smoke-induced DNA damage. MDA production and correlated DNA adduct (M1dG) formation in ethanol-exposed lung and/or derivative pulmonary cells have not been thoroughly investigated. Because the CYP2E1 pathway of ethanol metabolism is involved in the formation of MDA, there is promise in studying the role of specific inhibitors of CYP2E1 on DNA damage, MDA production, and M1dG adduct formation.
Our previous studies show a synergistic increase in MDA levels in lung lavage fluids when mice were fed alcohol and exposed to whole body cigarette smoke cigarette in comparison to either alone (84). This paper focuses on the effect ethanol consumption and cigarette smoke exposure has on HBEC’s and murine lung tissue by quantifying biomarkers (MDA and M1dG) of ethanol and cigarette smoke induced pulmonary epithelium cell DNA damage. We hypothesize that co-exposure to alcohol and cigarette smoke to HBEC and mouse lung will result in the formation of high amounts of MDA, necessary for the production of M1dG adduct. We also hypothesize that DADS will decrease the formation of this adduct.

3.2 Materials and Methods

Cell Culture

Primary normal HBEC were cultured in bronchial epithelial cell growth medium (BEGM, Lonza, Allendale, NJ) supplemented with 2 µg/mL of amphotericin (MP Biomedicals LLC, Solon, OH). Cells were plated on Type I collagen (Vitrogen 100, Collagen Biomaterials, Palo Alto, CA) coated tissue culture dishes at 37°C in a humidified, 5% CO₂ atmosphere. Cells were passaged once a wk at 1:3. Cells between the 2nd and 5th passage were used for experiments.

Cigarette Smoke Extract Preparation

Cigarette smoke extract (CSE) was prepared in accordance with Wyatt et al. 1999 (280). CSE was freshly prepared immediately before all experimental procedures. The cigarettes used were obtained from the University of Kentucky Tobacco-Health Research Division. A cigarette was connected to a peristaltic pump apparatus (Bentley Laboratories LLC, Edison, NJ), pyrolyzed, and the smoke was bubbled through 25 ml of sterile phosphate buffered saline (PBS; pH 7.4). One cigarette per 25 ml volume was used. The CSE in PBS was then sterile-filtered and diluted to 5% in BEGM (Lonza) as a percentage of the total volume.
Comet Assay

Comet assay was performed using a comet assay kit (Trevigen, Gaithersburg, MD) with slight modifications. Briefly, cells were cultured in 6-well plates, treated for 3 h with different conditions, and harvested. The cells were then pelleted and resuspended with cold PBS at 1 x 10^5 cells/ml. The cell suspension (50 µL) was mixed with 450 µl of low melting point agarose, and 50 µl of this mixture was pipetted over a sample area of comet slides. The slides were placed flat in a 4°C humidity chamber for 30 min, after which they were immersed in cold lysis solution provided in the kit and left at 4°C overnight. The slides were then transferred into alkaline unwinding solution (200 mM NaOH, 1 mM EDTA, pH 13) and incubated at room temperature for 60 min. The slides were then horizontally placed in an electrophoresis apparatus (Life Technologies Inc. Gaithersburg, MD) containing alkaline electrophoresis solution (200 mM NaOH, 1 mM EDTA pH 13) and run at 21 volt/cm for 30 min. After fixing with 70% ethanol for 5 min, slides were washed twice in distilled water, air-dried and stained with SYBR® gold staining solution for 30 min at 4°C. Cells were then viewed by epifluorescence microscopy (Nikon Eclipse E800, Melville, NY) and photographed with a digital camera (Nikon Digital Sight) under 4X magnifications. The measurement was performed using a public domain PC-image analysis program CASP software to analyze olive tail moment (OTM). An average of 70 cells were scored per sample each time.

Animal Exposure Model

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Female C57BL/6 mice (8-10 week old) were obtained from Jackson Laboratories (Bar Harbor, Maine), housed in-group cages, and fed commercial rodent chow and water ad libitum for 1 week. The mice were randomly assigned to 8 treatment groups (sham, alcohol, smoke, alcohol + smoke, DADS control, alcohol + DADS, smoke + DADS, and alcohol + smoke + DADS). Mice receiving DADS diet were fed 1 µM
DADS/g of their feed. All mice were weighed weekly. Alcohol feeding was performed as previously described in McCaskill et al. (281). Mice receiving alcohol were given increasing concentrations of alcohol in water over a 1-week period until the target concentration of 20% was reached using the Meadows-Cook model (282, 283). Mice in the alcohol group were given 10% ethanol (wt/vol) for 2 days, 15% ethanol (wt/vol) for 5 days, and 20% ethanol (wt/vol) for 7 week. Mice in the matched control group were given water from the same source without alcohol.

Cigarette smoke exposure was performed as previously described in McCaskill et al. 2011 (84) and Simet et al. 2010 (284). Briefly, cages containing C57BL/6 mice were placed in the exposure chamber of a Teague small animal whole body smoke exposure system (Model TE-10; Teague Enterprises, Davis, CA). Animals were exposed to a mixture of mainstream and side stream cigarette smoke via inhalation from 60 R1 reference cigarettes (Lexington, KY) at 150 mg/m$^3$ total smoking particles for 3 hr/day, 5 days/week, for up to 5 week. Mice receiving cigarette smoke were gradually brought to their target exposure over a period of 1 wk. Mice were exposed to smoke from 20 cigarettes for day 1, 30 cigarettes for day 2, 40 cigarettes for day 3, 50 cigarettes for day 4 and 5 and 60 cigarettes from day 5 to 5 week. Control animals were sham-exposed in chambers flowing room air.

**CYP2E1 ELISA**

CYP2E1 protein levels in the lung tissue homogenate were measured using a commercial ELISA kit (My Biosource, San Diego, CA). HBEC CYP2E1 protein was measured using a commercial ELISA (US Biological, Swampscott, MA) according to the manufacturer’s instructions. Briefly, HBEC were pretreated with 10 μM DADS for 1 hr and were further treated with ethanol, CSE and the combination of ethanol and CSE for 6 hr. After 6 hr, the media was removed and cells washed with PBS. The cells were then harvested with protease inhibitor cocktail (Sigma, St Louis, MO) diluted (1:10) in lysis buffer (tris-HCl, ethylene glycol, tetra-acetic acid, magnesium chloride pH 7.4) and centrifuged at 233g at 4°C and sonicated (to disrupt cell membranes).
Protein concentrations (mg/mL) were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) to standardize the ELISA results.

**ELISA for MDA**

MDA levels in the lung tissue homogenate and HBEC were measured using two different commercial ELISA kits (Cusabio, Wuhan, Hubei Province, China and US Biologicals, Swampscott, MA) according to the manufacturer’s instructions. Briefly, HBEC were pretreated with 10 µM DADS for 1 hr after which the cells were further treated with ethanol, CSE and the combination of ethanol and CSE for 6 hr. After 6 hr the media was removed and cells washed with PBS. Cells were then harvested with protease inhibitor cocktail, centrifuged at 4°C, sonicated, and protein measured as above before performing the ELISA.

**ELISA for M1dG**

Confluent HBEC were pretreated with 10 µM DADS for 1 hr and then further treated with ethanol, CSE and the combination of ethanol and CSE for 6 hr. After 6 hr the media was removed and washed with PBS. The cells were then harvested in lysis buffer containing protease inhibitor cocktail, centrifuged, and sonicated as described above before conducting the ELISA for M1dG. Briefly, polystyrene 96-well flat-bottomed plates (4 HBX; Thermo Scientific, Waltham, MA) were coated overnight at 4°C with 100 µl with samples (homogenized lung tissue or cell lysates in lysis buffer). Mouse anti-M1dG antibody was a kind gift of Dr. Lawrence Marnett (Vanderbilt University) and was conjugated to horseradish peroxidase (HRP) with a Lightning-LinkTM HRP Conjugation Kit (Innova Bioscience, Babraham, UK). Purified M1dG (Also kindly supplied by Dr. Lawrence Marnett) was added to mouse M1dG antibody conjugated with horseradish peroxidase (1:1000 in in PBS) to make final concentration of 40 ng/mL and incubated overnight in 4°C by gentle mixing in a shaker. After 24 hr the M1dG standard-antibody complex was further serially diluted (2 fold) with PBS for the standard curve. The plate, which was incubated
overnight with 100 µL of sample, was further washed with buffer (0.5 % PBS tween) and then the serially diluted standard was added to the standard wells in the 96-well plate. Mouse M1dG antibody conjugated with HRP (1:1000 in PBS) was added to the sample wells and incubated for another 3 hr at RT. After 3 hr, the plate was washed 4 times with wash buffer and developed with 100 µL tetramethylbenzidine substrate (Sigma, St Louis, MO) in the dark at room temperature before halting the color reaction with 8 M H₂SO₄. Plates were read at 450 nm using a plate reader (Molecular Devices, Sunnyvale, CA) to determine optical density.

**Statistical analysis**

All quantitative experiments were performed in triplicate. All data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA) and represented as mean ± standard error. Data were analyzed for statistical significance using one-way ANOVA followed by Tukey post hoc testing between each condition group. Significance was accepted at the 95% confidence interval.

### 3.3 Results

**Effect of ethanol and cigarette smoke exposure on single strand DNA damage**

Alcohol and cigarette smoke-induced single strand DNA damage was measured using COMET assay in primary HBEC (Fig. 4). Hydrogen peroxide was used as a positive control. Single strand DNA damage was measured using olive tail moment (OTM), which is the percentage of DNA in the tail and tail moment length from the center of the head to center of the tail. OTM increased significantly in the ethanol treatment group in comparison to the control group. There was also a significant increase in OTM when the cells were exposed to 5% cigarette smoke extract (CSE). Diallyl disulfide (DADS) treatment significantly reduced OTM when cells were treated with 10 µM DADS in combination with 80 mM ethanol. Similar results were also seen when cells were
treated with ethanol and 5% CSE in combination with DADS but no such effect was seen when cells were treated with CSE in presence of DADS.

Figure 4. Effect of ethanol exposure on single strand DNA damage measured by comet assay in HBEC.

Monolayers of human bronchial epithelial cells (HBEC) were pretreated for 1 hr with diallyl disulfide (DADS; 10 µM) and further treated for 3 hr with ethanol (EtOH; 80 mM), cigarette smoke extract (CSE; 5%) and co-exposure condition. Hydrogen peroxide (H₂O₂; 0.1 mM) was used as positive control. Exposure to ethanol caused significant DNA damage, which was reduced significantly by DADS exposure. Values represent mean ± S.E.M. of (100-150) cells scored. Experiment was performed 3 independent times.
Effect of ethanol and cigarette smoke exposure on CYP2E1 levels in HBEC and C57BL/6 mice whole lung tissue homogenate

Alcohol is known to induce CYP2E1 levels, therefore we measured the CYP2E1 levels in HBEC and mouse lung tissue homogenate of C57BL/6 mice fed with ethanol *ad libitum* in water and exposed to whole body cigarette smoke. In addition, some mice were fed a DADS diet. We found a significant increase in the CYP2E1 levels in the ethanol exposed HBEC in comparison to control which was not observed in mice exposed to 5% CSE. As expected, there was no significant difference between ethanol only exposed and cigarette smoked and ethanol co-exposed mice on CYP2E1 levels. But when cells were treated with DADS in combination with ethanol, there was a significant reduction in the CYP2E1 level (Fig. 5A). Likewise, we observed a similar result in mice fed ethanol and exposed to cigarette smoke along with DADS diet (Fig. 5B).

![Figure 5](image)

**Figure 5. Effect of ethanol and cigarette smoke on CYP2E1 levels in HBEC and C57BL/6 mice whole lung tissue homogenate.**

Human bronchial epithelial cells (HBEC) lysate and crude homogenates of mouse lung tissue were assayed using a sandwich ELISA. HBEC were pretreated for 1 hr with diallyl disulfide (DADS; 10 µM) and further exposed with ethanol (EtOH; 80 mM), cigarette smoke extract (CSE; 5%) and co-exposure condition for 6 hr and assayed using sandwich ELISA for CYP2E1 levels (A). Mice consumed EtOH *ad libitum* in water and were exposed to whole body cigarette smoke. Exposure to ethanol significantly induced CYP2E1 levels, which were reduced significantly by DADS.
supplement (B). Values represent mean ± S.E.M. of n=4 mice per condition and n=3 independent experiments for HBEC.

**MDA levels in HBEC and lung tissue exposed to ethanol, cigarette smoke, and diallyl disulfide**

We measured MDA, a marker of lipid peroxidation in HBEC and mouse lung tissue homogenate of C57BL/6 mice fed with ethanol *ad libitum* in water and exposed to whole body cigarette smoke, using a sandwich ELISA (Fig. 6A). We observed a significant increase in MDA levels when cells were treated with 80 mM ethanol for 6 hr. There was no significant increase in MDA levels when cells were exposed to 5% CSE alone. Co-exposure with ethanol and CSE did increase the MDA levels, but not significantly. Treatment with 10 µM DADS lowered the MDA level significantly in ethanol treated cells. This reduction was not observed in ethanol and cigarette smoke extract co-treatment. In mouse lung tissue homogenate (Fig. 6B), there was a significant increase in MDA levels in mice fed ethanol. We also observed a significant increase in MDA levels in mice fed ethanol and exposed to cigarette smoke. DADS supplement significantly lowered MDA levels in ethanol-fed mice. A similar effect was observed in mice fed ethanol and exposed to cigarette smoke when given DADS supplement. No such effect was seen on cigarette smoke only exposed mice.
**Figure 6. Effect of ethanol and cigarette smoke on MDA levels in HBEC and C57BL/6 mice whole lung tissue homogenate.**

Lysate of human bronchial epithelial cells (HBEC) were pretreated for 1 hr with diallyl disulfide (DADS; 10 µM) and further exposed to ethanol (EtOH; 80 mM), cigarette smoke extract (CSE; 5%) and co-exposure condition for 6hr and crude homogenates of mouse lung tissue were assayed for malondialdehyde (MDA) levels using a sandwich ELISA. Ethanol alone significantly increased MDA levels. Exposure to ethanol and cigarette smoke significantly increased MDA levels in tissue homogenate, which was reduced significantly by DADS supplement. Values represent mean ± S.E.M. of n=4 mice and n=3 independent experiments for HBEC.

**M1dG levels in HBEC and lung tissue exposed to ethanol, cigarette smoke, and DADS**

HBEC treated with ethanol exhibited significantly high M1dG adduct level in comparison to control (Fig. 7A). A similar result was observed when cells were treated with ethanol and 5% CSE, but we did not see any synergistic increase in the adduct levels. Interestingly, there was no significant increase in the M1dG adduct levels when cells were exposed to CSE alone. Treatment with 10 µM DADS along with ethanol significantly reduced the adduct levels. A similar result was observed when cells were co-exposed to ethanol and 5% CSE along with DADS. In mouse lung tissue homogenate (Fig. 7B) there was a significant increase in adduct level in mice fed ethanol ad libitum in water. We also observed a significant increase in adduct level in mice fed with ethanol and exposed to whole body cigarette smoke. Again, no synergy was observed. However, co-exposed mice fed DADS in their diet had a significant reduction in the adduct level.
in their lung tissue. A significant reduction in adduct level was also seen when ethanol-fed mice were given DADS in their diet.

Figure 7. Effect of ethanol and cigarette smoke on M1dG levels in HBEC and C57BL/6 mice whole lung tissue homogenate.

Lysate of human bronchial epithelial cells (HBEC) were pretreated for 1 hr with diallyl disulfide (DADS; 10 µM) and further exposed to ethanol (EtOH; 80 mM), cigarette smoke extract (CSE; 5%) and co-exposure condition for 6hr and crude homogenates of mouse lung tissue were assayed using indirect ELISA for M1dG levels. Ethanol alone and in combination with cigarette smoke significantly increased M1dG levels. Ethanol exposure significantly increased M1dG levels, which were reduced significantly by DADS supplement. Values represent mean ± S.E.M. of n=4 mice and of n=3 independent experiments for HBEC.

3.4 Discussion

In recent years researchers have focused on the effects of alcohol consumption and smoking on the lungs, but few studies have been conducted on the role of different types aldehydes formed in the lung tissue. Several studies have been conducted investigating the role of oxidative stress on pulmonary health and most of these studies have suggested reactive oxygen species as the main contributor to oxidative injury (285). Alcohol is primarily converted to acetaldehyde by both alcohol dehydrogenase (ADH) and the CYP2E1 pathway (149). In addition to converting ethanol
to acetaldehyde, ethanol metabolism via the CYP2E1 metabolic pathway leads to the formation of ROS, which reacts, with cell membrane lipids leading to lipid peroxidation. This lipid peroxidation leads to the generation of reactive aldehydes such as MDA (286). Many studies have shown that aldehydes such as acetaldehyde and MDA, which are formed due to alcohol metabolism can lead to the formation of adducts to both proteins and nucleotides (287). Acetaldehyde and MDA can react together with nucleophilic proteins to form stable hybrid adducts known as MDA-acetaldehyde (MAA) adducts (232).

Proteins are MAA-adducted in the lungs of mice under conditions of very high concentrations of both acetaldehyde and MDA observed only during co-exposure to both alcohol and cigarette smoke (84). However, in the current study, we propose a model (Fig. 8) in which CYP2E1-mediated metabolism of alcohol alone is the major pathway for the formation of M1dG adducts in the lung.

![Proposed model of M1dG adduct formation.](image)

**Figure 8. Proposed model of M1dG adduct formation.**

CYP2E1 mediated metabolism of alcohol leads to generation of reactive oxygen species (ROS). ROS enhances lipid peroxidation leading to the generation of malondialdehyde (MDA), which can adduct to the guanosine base of DNA to form a M1dG adduct. Inhibition of CYP2E1 by diallyl disulfide (DADS) leads to reduction in both MDA levels and M1dG adduct formation.
CYP2E1-mediated ethanol metabolism generates ROS, which can further lead to lipid peroxidation generating MDA, which can attach to the guanine base of DNA to form the M1dG adduct. Inhibition of CYP2E1 leads to a reduction in both MDA and M1dG adduct formation. Our study showed an increase in the single strand DNA damage in the HBEC when exposed to ethanol, but the highest DNA damage was seen in the co-exposure group. A similar result was observed in human intestinal epithelial Caco-2 cells (288) where cigarette smoke also led to a significant increase in DNA damage. Our result is consistent with studies done by Lui et al. in HBEC showing cigarette smoke extract-induced DNA damage (289). These findings suggest that a CYP2E1-independent effect on DNA damage occurs in response to cigarette smoke, likely in response to the known levels of oxidants contained in pyrolyzed tobacco. Our study also provides novel information about a specific malondialdehyde-mediated DNA adduct (M1dG) formed in the bronchial epithelial cells and lung tissue. High levels of M1dG adduct was measured in the alcohol-exposed group in comparison the smoke exposed group. This is consistent with results of our previous studies showing high level of MDA in the BALF fluid of alcohol fed mice in comparison to smoke exposed mice (84). Also, burning tobacco results in high acetaldehyde production and less MDA in comparison to alcohol only-exposed mice (84). In contrast to previous studies, there was no synergistic effect of alcohol and cigarette smoke on M1dG formation as previously in MAA adducted protein studies. Previous studies have shown that cigarette smoke and alcohol co-exposure is required to make sufficient acetaldehyde and MDA concentrations leading to MAA protein adduct formation in lung (88). In our study, we have found that exposure to alcohol results in sufficient MDA concentrations for M1dG adduct formation, suggesting that the pathway for the formation of these two adducts is likely different.

Our study also investigated the role of CYP2E1 in alcohol-mediated DNA damage, MDA and M1dG formation in cells and lung tissue. Consistent with previous studies, ethanol led to significant increase in CYP2E1 levels in both mouse tissue and HBEC (276, 290). Further inhibition of CYP2E1 with DADS led to the significant decrease in the single strand DNA
damage which was further supported by a decrease in MDA and M1dG adduct formation in both HBEC and mouse lung tissue. This result is in accordance with published results showing decreased oxidative stress and less MDA adduct formation by inhibition of CYP2E1 in human hepatoma VL-17A cell line and human hepatocytes by DADS (276, 277). These data provide evidence that CYP2E1 plays an important role in alcohol mediated oxidative stress and M1dG adduct formation.

3.5 Conclusion

In summary, alcohol consumption can lead to generation of reactive oxygen species resulting in lipid peroxidation and production of various gene toxic aldehydes such as MDA which can further lead to formation of various DNA (M1dG) adducts in lung tissue through a CYP2E1-mediated pathway. Heavy drinking of alcohol may lead to DNA damage suggesting a pathogenic role of MDA-derived DNA adducts in carcinogenesis, which can be reduced by the dietary consumption of DADS.
CHAPTER FOUR:
Malondialdehyde-Acetaldehyde (MAA) Adducted Surfactant Protein Alters Macrophage Functions through Scavenger Receptor A

4.1 Introduction

Alcohol intoxication compromises host defenses against bacterial infections as it leads to suppression of selected functions of the immune system (291). Individuals with alcohol use disorders (AUDs) are more susceptible to bacterial pneumonia than those without AUDs (292). In addition, persons with AUDs are two to three times more likely to smoke cigarettes (59, 228), leading to more frequent (293) and higher rates of cigarette smoking among AUDs than in the general population (294).

Macrophages are often called first responder phagocytes, ingesting and killing inhaled bacteria (295, 296). In the lung, alveolar macrophages make up approximately 93% of the macrophage population (297). They exhibit an inflammatory phenotype and secrete pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 and IL-1β, all of which participate in activation of antimicrobial mechanisms (298). Macrophages also produce reactive oxygen and nitrogen intermediates like nitric oxide and superoxide as a defense mechanism against microorganisms (299). Because macrophages play an important role in antimicrobial defense as well as to initiate, maintain, and resolve inflammation (296), any compromise in antimicrobial defense could lead to increased susceptibility to respiratory infection and unchecked inflammation resulting in cellular and tissue damage.

Because both acute and chronic alcohol consumption lowers the ability of phagocytes to clear diverse pathogens, alcohol consumption often leads to lower/compromised innate and acquired immune response (300). An increase in the quantity of alcohol consumption increases the risk for developing pneumococcal pneumonia (301). Increased susceptibility to pulmonary diseases such as pneumonia, acute respiratory distress syndrome and acute lung injury has also been shown to be associated with AUDs (297). It also known that alveolar macrophage function
is impaired after alcohol exposure in animal models (302). Similarly, cigarette smoking increases the number of alveolar macrophages and activates them to produce pro-inflammatory mediators and enzymes leading to inflammation and tissue damage (303). The ability of alveolar macrophages to phagocytize bacteria and to sense pathogen-associated molecular patterns (PAMPs) is also compromised in those who smoke cigarettes (304, 305), thus explaining the greater odds of developing invasive pneumococcal disease in people who have a smoking history (306, 307).

Reactive aldehydes such as acetaldehyde and malondialdehyde are formed during alcohol metabolism and oxidative stress in response to excessive alcohol consumption (149, 225). Acetaldehyde is also formed during the pyrolysis of tobacco and can also cause damage to protein and other molecules (84). Co-exposure to alcohol and cigarette smoke leads to the generation of high concentrations of acetaldehyde and malondialdehyde in the lung (88, 238). These aldehydes, once formed, react together covalently with proteins and generate MAA adducts, which possess immunogenic and pro-inflammatory properties (232). In lung, MAA covalently binds to surfactant protein D (SPD) secreted by type II epithelial cells to form an adducted protein called SPD-MAA (84).

Scavenger receptors are pattern recognition receptors expressed on the surface of macrophages (308). Among them, macrophage scavenger receptor A (SRA; CD204) has been most widely studied (309). It has the ability to bind and mediate the cellular uptake of modified lipoproteins (LDL)(310). In addition to modified LDL, this receptor binds to many different ligands such as maleylated bovine serum albumin (BSA), polyribonucleotides, polysaccharides, and aldehyde-modified proteins (235-237).

MAA-adducted proteins have been shown to be ligands for SRA on liver cells and bronchial epithelial cells (235, 237), but the effects of SPD-MAA on major macrophage functions and the role of SRA on such effects have not been evaluated extensively until now. Also MAA adducted to a SPD, functional surfactant protein in lung has been never used to study macrophage
functions. We, therefore, hypothesized that purified SPD-MAA formed in the lung of smokers and drinkers modulates macrophage functions primarily via SRA, one of the major receptor for MAA.

4.2 Materials and Methods

Materials

Protein kinase C (PKC) beta inhibitor LY316976 was purchased from Bio-Techne (Minneapolis, MN), PKC delta inhibitor Rottlerin and PKC alpha inhibitor Gö 6976 were purchased from Millipore (Billerica, MA). PKC zeta inhibitor myristolated PKC zeta inhibitory peptide and PKC epsilon inhibitor Ro 31-8220 were purchased from Enzo life science (Farmingdale, NY). Phorbol myristate acetate (PMA), nitro blue tetrazolium (NBT), fucoidan, fetuin and parthenolide were purchased from Sigma Aldrich (St Louis, MO). Lipopolysaccharide (LPS) was purchased from Fisher Scientific (Pittsburg, PA). Rat Anti-mouse SRA (2F8) was purchased from AbD Serotec (Raleigh, NC) and isotype negative control was purchased from Biolegend (San Diego, CA).

Cell culture and reagents

RAW 264.7 macrophages were purchased from American Type Cell Culture (ATCC, Rockville, MD) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37 °C in a humidified CO₂ incubator. Resident peritoneal macrophages were isolated from C57BL/6 wild type mice (Charles River, Wilmington, MA) and SRA knockout mice bred from homozygous SRA deficient mice (+/−) (B6.Cg-Msr1tm1Csk/J; Jackson Laboratory, Bar Harbor, ME) as described previously (311). All experimental animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. Briefly, peritoneal macrophages (PMs) were isolated by adherence to 24-well, flat-bottom, tissue culture-treated
polystyrene plastic plates in which \(1 \times 10^6\) peritoneal exudate cells were seeded per well and allowed to adhere for 2 h after which any non-adherent cells were washed away. At this point, the cells were 90% macrophages as confirmed by Diff-Quik™ Stain (Siemens Health Care Diagnostic Inc, Newark, DE). Isolated peritoneal macrophages were used for selective experiments.

**Malondialdehyde-acetaldehyde synthesis**

Human surfactant protein D (SPD) adducted to MAA (SPD-MAA) was prepared as previously reported (88). Briefly, approximately 1–1.5 mg/mL of SPD was incubated with 1.0 mM acetaldehyde and 1.0 mM MDA in pyrogen-free PBS. The pH was brought to 7.4, and maintained at 37 °C for 72 hr. At the end of incubation, the reaction mixture was exhaustively dialyzed against pyrogen-free phosphate buffer solution for 24 hr at 4 °C. The endotoxin level in the MAA-SPD was measured by limulus assay and was below the limit of detection.

**Pro-inflammatory cytokine release (TNFα and IL-6)**

To identify the role of SPD-MAA on pro-inflammatory cytokine release, RAW 264.7 and resident peritoneal macrophages (PMs) were treated with different concentrations of SPD-MAA (10-200 µg/mL) for different times (6-48 hr). The supernatant was then collected and stored at -80 °C for later analysis. To block SRA, RAW 264.7 macrophages were pre-incubated with fucoidan (50 µg/mL) for 72 hr or anti-SRA (10 µg/mL) for 24 hr followed by addition of SPD-MAA for another 6 hr.

**PKC isoform inhibition**

To identify the role of PKC on MAA adduct-mediated pro-inflammatory cytokine release, RAW 264.7 macrophages were pre-incubated with different isoform-specific inhibitors to PKC beta (LY316976), PKC delta (Rottlerin), PKC zeta (myristolated PKC zeta inhibitory peptide), PKC
alpha (Gö 6976) and PKC epsilon (Ro 31-8220) at 1 µM concentration for 1 hr followed by addition of 200 µg/mL of SPD MAA for another 6 hr. Resident peritoneal macrophages (PMs) were incubated with PKC alpha isoform inhibitor, Gö 6976 (1 µM) for 1 hr followed by SPD-MAA treatment for 6 hr. After 6 hr, supernatant was collected and later analyzed for cytokines levels.

**NF-kB inhibition**

To identify the role of NF-kB in MAA adduct-mediated pro-inflammatory cytokine release, macrophages were pre-incubated with 1 µM parthenolide for 1 hr followed by 200 µg/mL SPD-MAA treatment for another 6 hr. After 6 hr, supernatant was collected and later analyzed for pro-inflammatory cytokines.

**ELISA**

TNFα and IL-6 levels in the supernatant were measured by ELISA according to the manufacturer’s instructions (R & D Systems, Minneapolis, MN).

**Phagocytosis**

Phagocytosis of zymosan particles was assayed using a commercially available kit (Cell Biolabs, San Diego, CA). First, the macrophages were treated with 200 µg/mL SPD-MAA for 3 hr followed by addition of zymosan particles (5 X 10⁶) for (0-60) min and later analyzed for phagocytosis of the zymosan by measuring the optical density at 430 nm according to manufacturer instructions. For PMs, the macrophage were treated with 200 µg/mL SPD-MAA for 3 hr followed by addition of zymosan particles (5 X 10⁶) for 30 min.
Superoxide ion production

PMA stimulated superoxide ion production

Superoxide ion production was assayed using previously established NBT method as previously described (312). RAW 264.7 macrophages were pre-treated with 200 µg/mL SPD-MAA for 24 hr after which, PMA (300 ng /mL) in NBT solution (1mg/mL) was added to the macrophages. After 1 hr, macrophages were washed with phosphate buffer saline (PBS, pH 7.4) twice and later fixed with 100% methanol. The plate was then allowed to air dry for 10 min. After the complete removal of methanol, 120 µL of 2M potassium hydroxide was added followed by addition of 140 µL of 100 % dimethylsulfoxide and incubated at room temperature for 10 min. After 10 min, 200 µL of the solution was transferred to another 96-well plate and optical density was measured at 630nm (BioTek Instruments, Inc, Winooski, VT).

Opsonized zymosan stimulated superoxide ion production

Opsonized zymosan was prepared using a previously established method (313). First 10 mg of zymosan A (Sigma Aldrich, St Louis, MO) was opsonized with 1mL of normal mouse serum (Invitrogen, Frederick, MO) by incubating at 37°C for 30 min followed by washing with PBS (pH 7.4) twice. RAW 246.7 macrophages were pre-treated with 200 µg/mL SPD-MAA for 24 hr after which opsonized zymosan (1mg/mL) in NBT solution (1mg/mL) was added to the macrophages. After 30 min, macrophages were washed with PBS and superoxide ion production was measured as described above.

Nitrite production

Nitrite production was assayed by measuring nitrite [a stable degradation product of nitric oxide (NO)] in the supernatant of cultured RAW 264.7 macrophage using Griess reagent. Briefly, RAW 264.7 macrophages were pretreated with 200 µg/mL of SPD-MAA for 3 hr followed by treatment with 0.3 mg/mL zymosan (Sigma Aldrich, St Louis, MO) in Hams F-12 nutrient medium
containing 10% FBS. After 24 hr, supernatant was collected and nitrite level was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI).

**Real-time quantitative RT-PCR**

RAW 264.7 macrophages were treated with SPD-MAA (200 µg/mL) for (1-24 hr) and later total RNA was isolated from the macrophages using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The purity of RNA was checked by measurement of optical density (260/280) ratio using a NanoDrop spectrophotometer (Thermoscientific). cDNA was synthesized using 100 ng of template RNA and a TaqMan reverse transcription kit (Applied Biosystems, Austin, TX). Real-time PCR reactions were prepared using TaqMan Master Mix (Applied Biosystems) and primers and probe for SRA (Applied Biosystems; Mm 00446214_m1). Ribosomal (18S) RNA was used as an endogenous control. PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Threshold values were normalized to the expression of ribosomal RNA. Real-time PCR results are expressed as the fold change increase in comparison to media control.

**SRA surface expression**

RAW 264.7 macrophages were treated with SPD-MAA (200 µg/mL) for (1-24 hr). After treatment, macrophages were washed with ice-cold PBS followed by fixation with 3.7 % paraformaldehyde for 10 min at 4°C. After fixation, macrophages were washed with PBS followed by incubation with rat anti-SRA labeled with FITC and matched isotype control antibody at 4°C for 1 hr. After incubation macrophages were washed with PBS three times and the samples were analyzed by flow cytometry. Flow cytometry analysis was performed with FACS Calibur available in the UNMC Flow Cytometry Core (BD Biosciences, San Jose, CA).
Statistics

All data were analyzed using GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego CA) and represented as mean ± SE. Data were analyzed for statistical significance using Student’s t-test and both one-way and two-way ANOVA employing Tukey’s multiple comparison post-test corrections. A p-value less than 0.05 was regarded as statistically significant.

4.3 Results

Cell viability

Cell viability was assessed by trypan blue exclusion method as described previously (314). No cell death was observed for different concentrations of SPD-MAA over a period of 6-48 hr as measured by trypan blue exclusion method. Also, the PKC alpha inhibitor, NF-κB inhibitor, fucoidan and anti-SRA did not affect cell viability (data not shown).

SPD-MAA exposure induces pro-inflammatory cytokine TNFα release

To determine whether exposure to SPD-MAA induces pro-inflammatory cytokine TNF-α release, RAW 264.7 macrophages were exposed to 200 µg/mL of SPD-MAA for different time points (6-48 hr). RAW 264.7 macrophages produced TNF-α in response to SPD-MAA in a time-dependent manner. A significant increase (p < 0.0001) in TNF-α release was observed as early as 6 hr in comparison to media control (Fig. 9A). Additionally, a dose-dependent increase in TNF-α release was observed in response to SPD-MAA. TNF-α release was significantly increased (p < 0.001) following exposure to 100-200 µg/mL of SPD-MAA at 6 hr (Fig. 9B). No significant increase in TNF-α release was observed when exposed to non-adducted lung surfactant protein SPD (Fig. 9B). These data demonstrate that SPD-MAA induces TNF-α release from RAW 264.7 macrophages in both a time- and concentration-dependent manner. Similarly, in peritoneal
macrophages (PMs) isolated from WT mice, we observed a significant dose-dependent (p < 0.0001) increase in TNF-α release at 6 hr when treated with 200 μg/mL of SPD-MAA (Fig. 9C). No such response was observed in PMs from SRA KO mice (Fig. 9C).

Figure 9. Effect of SPD-MAA on pro-inflammatory cytokine TNF-α release.

TNFα release from RAW 264.7 macrophages was measured after treatment with 200 μg/mL SPD-MAA for 6, 24 and 48 hr (A). After treatment, supernatant was collected and TNF-α level measured by ELISA. SPD-MAA induced TNF-α release from RAW 264.7 (B) and wild type peritoneal macrophages (C) in concentration dependent manner at 6 hr. No such effect was seen in SRA KO mice (C). RAW 264.7 macrophages were pretreated with 50 μg/mL of fucoidan (D).
for 72 hr or 10 µg/mL of Anti-SRA (E) for 24 hr followed by treatment of SPD-MAA for 6 hr. Values are the mean ± SEM of n=3 of three independent experiments.

In addition, significantly less TNF-α release was observed at 100 µg/mL (p < 0.05) and 200 µg/mL (p < 0.0001) of SPD-MAA treatment in SRA KO mice in comparison to WT mice (Fig. 9C). Pretreatment blocking with fucoidan, a ligand for scavenger receptor A, significantly reduced (p < 0.001) SPD-MAA-induced TNF-α release in RAW 264.7 macrophages (Fig. 9D). No reduction in SPD-MAA stimulated TNF-α release was observed when macrophages were treated with fetuin, a glycoprotein negative control for fucoidan (data not shown). Treatment with SRA blocking antibody (2F8) significantly reduced (p < 0.001) SPD-MAA stimulated TNF-α release (Fig. 9E). No reduction in SPD-MAA stimulated TNF-α release was observed when macrophages were treated with isotype-matched negative control, IgG (data not shown).

**SPD-MAA exposure induces pro-inflammatory cytokine IL-6 release**

To determine whether exposure to SPD-MAA induces pro-inflammatory cytokine IL-6 release, PMs from both WT and SRA-/- mice were exposed to different doses of SPD-MAA for 6 hr. A significant increase in IL-6 was detected at the highest concentration of SPD-MAA (200 µg/mL) (p < 0.0001)(Fig. 10). No such increase was observed in PMs from SRA-/- mice. We also observed a significant attenuation of IL-6 release at 200 µg/mL of SPD-MAA (p < 0.0001) treatment in PMs from SRA KO in comparison to WT mice (Fig. 10). No time or dose-dependent increase in IL-6 was observed when RAW 264.7 macrophages were exposed to SPD-MAA (200 µg/mL) for up to 24 hr (data not shown).
Peritoneal macrophages (PMs) from wild type (WT) and SRA KO mice were treated 200 µg/mL SPD-MAA for 6 hr. After treatment, supernatant media was collected and IL-6 levels measured by ELISA. SPD-MAA induced IL-6 release from PMs from WT mice at highest concentration at 6 hr. No such effect was seen on PMs from SRA KO mice. Values represent mean ± SEM of n=3 of three independent experiments.

**Figure 10. Effect of SPD-MAA on pro-inflammatory cytokine IL-6 release.**

Peritoneal macrophages (PMs) from wild type (WT) and SRA KO mice were treated 200 µg/mL SPD-MAA for 6 hr. After treatment, supernatant media was collected and IL-6 levels measured by ELISA. SPD-MAA induced IL-6 release from PMs from WT mice at highest concentration at 6 hr. No such effect was seen on PMs from SRA KO mice. Values represent mean ± SEM of n=3 of three independent experiments.

**Effect of SPD-MAA on phagocytosis of zymosan particles**

To determine the role of SPD-MAA on phagocytic function, RAW 264.6 macrophages were incubated with 200 µg/mL SPD-MAA for 3 hr followed by addition of zymosan particles for 0-60 min. Treatment with SPD-MAA significantly decreased the phagocytosis of zymosan particles at 30 min (p < 0.0001) and 60 min (p < 0.0001) in comparison to their respective media control (Fig. 11A). No reduction was observed when macrophages were treated with non-adducted SPD only (Fig. 11A). A similar result was observed in PMs from WT mice treated with 200 µg/mL SPD-MAA for 3 hr. In PMs, effective phagocytosis of zymosan particles was observed at 30 min in the media control treatment (p < 0.0001) while SPD-MAA treatment significantly reduced (p < 0.0001) phagocytosis in WT mice at 30 min (Fig. 11B). Importantly, no further SPD-MAA-
mediated reduction in phagocytosis was observed in SRA KO mice. Similar to RAW 264.7 macrophages, non-adducted SPD treatment did not diminish phagocytosis of zymosan particles by PMs from WT mice at 30 min (Fig. 11B).

![Graph A](image)

**Figure 11. Effect of SPD-MAA on phagocytosis of zymosan particles.**

RAW 264.7 macrophages and peritoneal macrophages (PMs) were treated with 200 µg/mL of SPD-MAA for 3 hr followed by addition of zymosan particles for (0-60) min. Phagocytosis of the particles was detected as described in methods. SPD-MAA treated significantly reduced the phagocytosis of particles by RAW 264.7 macrophage (A) and PMs (B) from wild type (WT) mice (white bars). No such effect was seen in PMs from SRA KO mice (B; black bars). Values represent mean ± SEM of n=3 independent experiments.
Effect of SPD-MAA on superoxide ion and nitrite release

To determine the role of SPD-MAA on bactericidal function, superoxide ion and nitrite ion release was measured. RAW 264.6 macrophages were pre-incubated with 200 µg/mL SPD-MAA for 24 hr followed by addition of PMA (300 ng/mL) for 1 hr or opsonized zymosan for 30 min. SPD-MAA significantly reduced superoxide ion release from RAW 264.7 macrophages in response to zymosan (p < 0.001)(Fig. 12A). A similar result was observed in response to PMA (p < 0.001)(Fig. 12B). Pre-treatment with non-adducted SPD had no such effect (Fig. 12A and 12B).

To measure nitrite release, RAW 264.7 macrophages were pre-treated with 200 ug/mL of SPD-MAA for 3 hr followed by the addition of zymosan (0.3 mg/mL) for another 24 hr. SPD-MAA significantly reduced (p < 0.01) nitrite release in response to zymosan (Fig. 12C). No reduction in nitrite release was observed when exposed to equal concentration of SPD (Fig. 12C).
Figure 12. Effect of SPD-MAA on nitrite and superoxide ion release.

RAW 264.7 macrophages were pretreated with 200 µg/mL SDP-MAA for 24 hr followed by treatment with PMA (300 ng/mL) for 1 hr (A) or opsonized zymosan (1mg/mL) for 30 min (B). After treatment time superoxide ion was measured using NBT method. For nitrite release, RAW 264.7 macrophages were pretreated with 200 µg/mL SDP-MAA for 3 hr followed by treatment with zymosan (0.3 mg/mL) for another 24 hr. After 24 hr supernatant was collected and nitrite level measured (C). Values represent mean ± SEM of n=3 independent experiments.
Effect of SPD-MAA on SRA expression

To determine the effect of SPD-MAA on SRA message expression, RAW 264.6 macrophages were treated with 200 µg/mL SPD-MAA for 1, 3, 6, and 24 hr and later analyzed for SRA mRNA expression. A significant up regulation of SRA mRNA expression was observed at 6 hr (p < 0.0278) (Fig. 13A). At 24 hr, however, SRA mRNA expression returned to baseline. No such effect on SR-A mRNA was seen when RAW 264.6 cells were treated with SPD for 6 hr (Fig. 13A). To determine the effect of SPD-MAA on surface expression of SRA, RAW 264.6 macrophages were treated with 200 µg/mL SPD-MAA for 0-24 hr and analyzed for surface receptor expression by flow cytometry. No significant change in SRA surface expression was detected at 1, 3, 6, or 24 hr (Fig. 13B). No rapid transient change in SRA expression was observed at 0-30 min (data not shown).
**Figure 13. Effect of SPD-MAA on SR-A expression.**

RAW 264.7 macrophages were treated with SPD-MAA for (1-24 hr) and later analyzed for SRA message (A) expression by RT-PCR. Results represent fold change in SRA message expression from media control. RAW 264.7 macrophages treated with SPD-MAA (0-24 hr) and later analyzed for SRA surface receptor expression by flow cytometry (B). Values represent mean ± SEM of n=3 independent experiments.

**MAA-adducted protein binds to SRA and causes PKC activation**

To identify the role of PKC on SPD-MAA induced pro-inflammatory cytokine release, RAW 264.7 macrophages were incubated with different PKC isoform inhibitors at 1 µM concentration (315) for 1 hr before addition of 200 µg/mL SPD-MAA for 6 hr. PKC alpha inhibitor Gö 6976 pretreatment significantly decreased TNFα release from RAW 264.7 macrophages in response to SPD-MAA (p < 0.001)(Fig. 14A). No other PKC inhibitors affected TNF-α release (Fig. 14A). A
similar result was observed when PMs from WT mice were pretreated with PKC alpha inhibitor Gö 6976 for 1 hr (p < 0.05)(Fig. 14B). In addition to TNFα, pretreatment with Gö 6976 also significantly decreased IL-6 release from PMs macrophages from WT mice in response to SPD-MAA at 6 h (p < 0.001)(Fig. 14C).

**Figure 14. Effect of PKC isoform inhibitors on SPD-MAA stimulated TNF-α and IL-6 release.**

RAW 264.7 macrophages (A) were pretreated with different PKC isoform inhibitors at 1 µM concentration for 1 hr before addition of 200 µg/mL SPD-MAA for 6 hr (A). PMs from WT mice were pretreated with PKC alpha inhibitor Gö 6976 (1µM) for 1 hr and later treated with 200 µg/mL SPD-MAA or SPD for 6 hr. After treatment, supernatant was collected and TNFα (A, B) or IL-6 (C) levels were measured by ELISA. Values are the mean ± SEM of n=3 independent experiments. Legend same for all 3 figures.
MAA-adducted protein binds to SRA and causes NF-κB activation

To identify the role of transcription factor NF-κB on SPD-MAA induced pro-inflammatory cytokine release, RAW 264.7 and PMs were incubated with the NF-κB inhibitor parthenolide at 1 µM concentration for 1 hr before addition of 200 µg/mL SPD-MAA for 6 hr. A significant reduction in TNF-α release was observed in both RAW 264.7 macrophages (p < 0.001; Fig. 15A) and PMs (p < 0.01; Fig. 15B). In addition to TNF-α, pretreatment with NF-κB inhibitor, parthenolide, significantly reduced IL-6 release from PMs at 6 hr in response to SPD-MAA (p < 0.001; Fig. 15C).
Figure 15. Effect of NF-κB inhibitor on SPD-MAA stimulated TNF α and IL-6 alpha release.

RAW 264.7 macrophages (A) were incubated with NF-κB inhibitor (parthenolide) at 1 µM concentration for 1 hr before addition of 200 µg/mL SPD-MAA for 6 hr (A). PMs from WT mice were pretreated parthenolide (1uM) for 1 hr and later treated with 200 µg/mL SPD-MAA for 6 hr SPD (B, C). After treatment, supernatant was collected and TNF-α (A, B) and IL-6 (C) levels were measured by ELISA. Values are the mean ± SEM of n=3 independent experiments. Legend same for all 3 figures.
4.4 Discussion

It is well established that macrophages play an important role in lung immunity. Any alteration in macrophage function could lead to lung inflammation, injury and infection. Aldehyde adducts have been shown to induce the release of pro-inflammatory cytokines and chemokines by Kupffer, endothelial, and stellate cells of the liver (316). Our study showed that SPD-MAA, an adduct that is formed in the lung during co-exposure to alcohol and cigarette smoke to a biologically relevant lung surfactant protein, activates macrophages to release pro-inflammatory cytokines TNF-α and IL-6, but not IL-1β. Previously, Hill et al. reported the release of the pro-inflammatory cytokine TNF-α in a purified rat heart endothelial cell culture (HEC) when stimulated with MAA-adduct (247). Pro-inflammatory cytokine elevations in the lung of human subjects with alcohol use disorders (AUDs) and who smoke cigarette (307) could be due in part to formation of MAA adduct in their lung. Thiele et al. also reported that MAA adducts induced secretion of several cytokines and chemokines, including TNF-α, monocyte chemo-attractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) from liver endothelial cells (317). We observed a significant increase in IL-6 release from peritoneal macrophages (PMs) at the highest concentration of SPD-MAA, but not from RAW 264.7 macrophages, a cell line. This could be due to differences in responses between primary cells and an immortalized cell line. Regardless, peritoneal macrophages and RAW 264.7 macrophages have been extensively used in lung studies (318-324).

Interestingly, our data also demonstrate that SPD-MAA activates macrophages to release selective pro-inflammatory cytokines. Hsu et al. showed that Ox-LDL, a ligand for scavenger receptor, upregulated TNF-α production, but not IL-1β secretion in the murine macrophage cell line, J774A.1 (325). One explanation for our result could be that SPD-MAA binding to scavenger receptor may activate protein kinase C and NF-kB, resulting in TNF-α and IL-6 release; whereas, another signaling pathway, mitogen-activated protein kinase (MAPK), may be involved for IL-1β release (325). Even though TNF-α, IL-6 and IL-1β are the major pro-inflammatory cytokines
released by macrophages, our results indicate that TNF-α is the most important. Our study also showed higher levels of TNF-α release from peritoneal macrophages than from RAW 264.7 macrophages. Also, unlike TNF-α, only very high concentrations of SPD-MAA stimulated IL-6 release from peritoneal macrophages while RAW 264.7 macrophages were unresponsive. Differences in both cytokine expression and response between primary macrophages and immortalized macrophage cell lines could explain this result (326). In contrast to our results, BSA-MAA did not stimulate IL-6 release from bovine bronchial epithelial cells (238), which could be due to difference in the species from which the cells were derived.

Our study is the first to report that SPD-MAA significantly reduces the phagocytic function of macrophages. A reduction in phagocytic activity was previously reported when RAW 264.7 macrophages were treated with n-butanol (327). Additionally, several studies involving mice and human subjects have reported that alcohol inhibits monocyte and macrophage phagocytic function as well as antimicrobial activity in vitro (328, 329). Cigarette smoke has also been reported to reduce alveolar macrophage phagocytic function (304, 330). Thus, the increased susceptibility to respiratory infection in subjects with AUD who also smoke could be compounded by MAA adduct-mediated impairment of macrophage phagocytic function in the lung.

Oxidative burst involving the release of superoxide (O$_2^-$) and nitrite ion (NO$_2^-$) contributes to the ability of the activated macrophage to inhibit growth and viability of a variety of pathogens. An insufficient release of these mediators may increase host susceptibility to a subsequent infection (331). Our results showed a significant reduction in PMA and opsonized zymosan-stimulated superoxide ion release when RAW 264.7 macrophages were pretreated with SPD-MAA. A decrease in production of superoxide ion from PMA and opsonized zymosan-stimulated alveolar macrophages in EtOH-fed rats was previously reported (332, 333). Similarly, cigarette smoke was reported to increase pulmonary bacterial burden in both in vivo and in vitro models (334). Alcohol-mediated reduction in LPS-stimulated nitric oxide release from
macrophages has been previously reported (327, 332, 335). Our study is the first one to report MAA adduct-mediated reduction in nitrite release in response to zymosan from macrophages.

Our data also demonstrate that scavenger receptor A is one of the major receptors for our observed responses to SPD-MAA in the macrophage. TNF-α and IL-6 release was significantly reduced in SRA−/− PMs in comparison to WT mice. This result suggests that the MAA adduct most likely binds to scavenger receptors and signals the release of cytokines as postulated by Tuma et al. (232). Thiele et al. also reported diminished responses to hen egg lysozyme (HEL) and HEL-MAA in SRA−/− mice when compared with WT control (317). A significant reduction in specific binding as well as uptake of AcLDL in SRA KO macrophages in comparison to WT macrophages has also been reported (336, 337). Our data also showed that pretreatment with fucoidan, a ligand that competes with SPD-MAA for scavenger receptor, or SRA blocking antibody significantly reduced SPD-MAA induced TNF-α release from RAW 264.7 macrophages. Previously, fucoidan has been shown to decrease SPD-MAA binding to bronchial epithelial cells (239). Furthermore, Berger et al. also reported that MAA-adducted proteins stimulate an inflammatory response in airway epithelial cells through binding to SRA (237). Thus, SRA is one of the major receptor for SPD-MAA in macrophages and is required to initiate intracellular signaling in response to SPD-MAA binding (325, 338).

We also report that exposure to SPD-MAA induced SRA mRNA expression. Our result is in accordance with previous published results utilizing other SRA ligands. Han et al. and Nikolic et al. reported marked induction of macrophage scavenger receptor mRNA expression on macrophages after exposure to lipoproteins such as OxLDL and AcLDL (339, 340). We found no change in surface expression of SRA in response to SPD-MAA treatment at any time point tested. Similarly, no change in SRA surface expression on macrophages occurs with low-density lipoprotein (LDL) (338, 341). Our result suggests that SPD-MAA may bind to the SRA on macrophage surface and initiate signaling mechanism involved in cytokine production. Not only does SPD-MAA binds to SRA but also induce expression of the receptor. No change in the
surface expression suggests that this receptor either does not internalize after SPD-MAA binding or is continually being replenished.

Our data suggest a role for PKCα, a calcium-dependent classical protein kinase C isoform, in pro-inflammatory cytokine release in response to SPD-MAA. PKCα specific inhibitor, Gö 6976, inhibited the production of TNF-α and IL-6 in response to SPD-MAA. Other PKC isoform-specific inhibitors failed to inhibit TNF-α release. Previously, SRA ligands such as AcLDL and fucoidan have been reported to induce protein tyrosine phosphorylation and PKC activity (239, 342). A specific role of PKC alpha has also been implicated for MAA-induced IL-8 release (238). Our results also indicate the role of transcription factor NF-κB in TNF-α and IL-6 release in response to SPD-MAA. Inhibition of NF-κB with parthenolide significantly reduced TNF-α and IL-6 release in response to SPD-MAA. Our result is in accordance with Janabi et al. who showed oxidized LDL, an SRA ligand, induces NF-κB activation leading to subsequent expression of pro-inflammatory genes (343). Other studies have also implicated PKCα as an important upstream kinase that activates NF-κB (315, 344).

Also in our studies we observed that 200 µg/mL SPD-MAA was required for a significant response. This dose was higher than that required for an in-vivo lung repeat-exposure study (50 µg /mL for 3 weeks)(88) or the amount of MAA formed in mouse lung after chronic alcohol and cigarette smoke exposure (500 ng/mL over 8 weeks)(84). This further suggests that a high concentration of MAA is required to get an in vitro response from macrophages in a short exposure period and that in vivo studies may model chronic exposure best.
Figure 16. Proposed model for SPD-MAA mediated effects on macrophage functions.

Hybrid adduct SPD-MAA in the lung binds to SRA expressed on the macrophage surface and modulates macrophage functions. SPD-MAA exposure decreases phagocytosis and superoxide ion release as well as increases pro-inflammatory cytokines TNF-α and IL-6 release. Using SRA competing ligand fucoidan, SRA ligand blocking antibody or knocking out SRA gene diminishes these modulations. PKC alpha inhibitor Gö 6976 and NF-κB inhibitor parthenolide inhibited pro-inflammatory cytokines release from macrophage in response to SPD-MAA.

A major limitation of our study is that our results are limited to RAW 264.7 and peritoneal macrophages. Because SPD is a lung defense protein shown to be the target of MAA adduction, primary lung macrophages would have been ideal to further confirm SPD-MAA effects observed in RAW 264.7 cells and PMs, but primary lung macrophages are difficult to obtain in sufficient
viable numbers to perform the extensive studies reported in the current study. So all study was
done in RAW 264.7 and peritoneal macrophages. Also it is important to note that MAA adduct
formation can involve a wide variety of target proteins and the MAA moiety has itself been
shown to be inflammatory and immunogenic (88). So several previous studies have utilized non-
bio logically relevant proteins such as BSA for lung and liver studies (88, 238, 345, 346). To
overcome the above-mentioned limitation, future in-vivo studies using WT and SRA KO mice is
necessary to further confirm results observed in both macrophages and airway epithelial cells.
This will also justify the role of SRA in SPD-MAA mediated lung effects.

4.5 Conclusion
To summarize, our current study demonstrates that MAA adducted proteins modulate certain
macrophage inflammatory and effector functions in RAW 264.7 and PMs (Fig. 16). Such
modulations may involve secretion of pro-inflammatory cytokines such as TNF-α and IL-6 as
well as compromised phagocytic and superoxide and nitrite ion release. Additionally, our study
also emphasizes the functional role of SRA in mediating the effects of SPD-MAA since in the
absence of SRA, MAA failed to reduce macrophage function. Our data also suggest that PKCα
and NF-κB play roles in MAA adduct-stimulated pro-inflammatory cytokine release by these
macrophages.
CHAPTER FIVE:
Malondialdehyde-Acetaldehyde (MAA) Adducted Surfactant Protein Induced Lung Inflammation is Mediated Through Scavenger Receptor A (SRA)

5.1 Introduction
People who abuse alcohol are likely to smoke cigarettes, and similarly, smokers are more likely to drink alcohol than non-smokers (58). Among individuals with alcohol use disorders (AUDs), smoking rates are estimated to be 90% and more than 70% of these individuals smoke at least one pack of cigarettes per day (347, 348). Well established as the major cause of all lung cancers, cigarette smoking is also a risk factor for respiratory tract infections like pneumonia and tuberculosis. Smoking is also the primary risk factor for chronic obstructive pulmonary disease (COPD)(349), projected to be the third leading global cause of death by 2030 (350). Because the chronic consumption of alcohol has a wide range of effects on lung function, it could increase the risks of pneumonia, acute respiratory distress syndrome (ARDS)(104) and COPD (351).

Although most ingested alcohol is metabolized in the liver, significant concentrations of ethanol reach the airway passages via the bronchial circulation (82). In the lung of chronic alcohol drinkers, airways can be continuously exposed to high concentrations of alcohol due to the “rain effect” (81). Generally, alcohol is metabolized by the alcohol dehydrogenase (ADH) pathway to generate acetaldehyde (82). In chronic alcohol consumption, however CYP2E1 pathway is induced, leading to oxidative stress, lipid peroxidation and generation of malondialdehyde (225, 352). In alcohol abusers who smoke cigarettes, even higher concentrations of reactive aldehydes are formed in the lung. Metabolism of alcohol through ADH and CYP2E1 leads to generation of acetaldehyde (AA) and malondialdehyde (MDA)(149). In addition, pyrolysis of tobacco generates high concentrations of AA (146). Cigarette smoke also induces
oxidative stress, leading to lipid peroxidation resulting in high concentrations of MDA (353). These aldehydes in lung, being highly reactive and electrophilic, bind to nucleophilic sites on proteins through a Schiff base reaction (85), leading to the formation of hybrid protein adducts. Unlike other individual aldehyde protein adducts, this hybrid malondialdehyde-acetaldehyde (MAA) adduct is very stable (232). The lungs of alcohol abusers who also smoke cigarettes are the ideal environment for the formation of MAA adduct (84). Lung surfactant protein D (SPD), produced by type II alveolar epithelial cells, is one target protein adducted by reactive aldehydes to form SPD-MAA (84). MAA adducts in lung have been shown to delay wound healing and increase protein kinase C-dependent IL-8 release as well as decrease cilia beating frequency (238). Additionally, MAA adducted protein instilled into lungs of mice produce inflammatory injury (88).

Scavenger receptor A (SRA/CD204), expressed largely on macrophages is also present on dendritic cells, endothelial cells, Kupffer cells, airway epithelial cells and vascular smooth muscle cells (338, 354). SRA plays an important, well-established role in atherosclerosis (236, 355, 356). SRA can bind to a broad range of ligands such as oxidized LDL, acetylated LDL, fucoidan, dextran sulfate and modified self proteins (357) as well as a number of conserved microbial structures, such as bacterial lipopolysaccharide and lipoteichoic acid (358). Based upon in vitro evidence, SRA may play an important role in innate immunity, and an affinity for modified lipids and pathogens might suggest its role in inflammation (359). SRA has been shown to be important in the uptake of the MAA adduct in bronchial epithelial cells (237) and liver sinusoidal endothelial cells (235). Absence of this receptor has been shown to reduce IL-8 release from mouse tracheal epithelial cells (237) as well as decrease antibody response to MAA-Alb (235). SRA has also been shown to be one of the primary receptors for MAA adduct on macrophages (360).

Our previous study in mice shows that MAA has a pro-inflammatory effect in lung after 3 weeks of instillation as an increase in the pro-inflammatory chemokine, keratinocyte
chemoattractant (KC; CXCL1) and lung lavage neutrophils was observed (88). Also, SRA binds MAA adducts primarily in both bronchial epithelial cells and macrophages in previous studies (88, 360). SRA is important for MAA adduct binding and plays an important role in MAA-mediated effects. Because the role of SRA in MAA adduct-mediated lung inflammation and injury has not yet been studied, we hypothesized that SRA has an important role in MAA-mediated lung inflammation, and that in the absence of this receptor, the effects of MAA in mouse lung is decreased. We show, for the first time, the role of SRA in MAA-mediated lung inflammatory effects in a mouse model.

5.2 Materials and Methods

Mice

WT C57BL/6 mice were purchased from the Charles River (Wilmington, MA) at 6–8 weeks of age and SRA knockout mice bred from homozygous SRA-deficient mice (+/+) (B6.Cg-Msr1tm1Csk/J; Jackson Laboratory, Bar Harbor, ME). All mice were housed in group cages and received standard rodent chow and water ad libitum for 1 week before the start of the experiment. Mice were monitored daily and weighed weekly. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Intranasal Instillation

The intranasal instillation was performed as previously described (88). Briefly, mice were first assigned to three treatment groups: saline, MAA adducted protein, and non-adducted protein control (SPD). All three treatments (saline, MAA and SPD) were sterile and free of endotoxin. For intranasal instillation each mouse was anesthetized using isoflurane inhalation and then each mouse was positioned with its head held back to make the nasal pathway vertical. 50 µl of the
treatment solution (sterile PBS or 50 \( \mu g/mL \) of MAA or SPD) was gently placed on the nasal openings with a pipette tip. After the instillation, each mouse was held in the same position to ensure complete inhalation and then monitored until it was awake and moving around normally. This procedure was done one time and also repeated daily for 1-3 weeks. None of the mice showed any sign of distress.

**Bronchoalveolar Lavage (BAL)**

After the instillation period, mice were euthanized by isoflurane overdose. The trachea was then exposed and a cannula inserted just below the larynx. The proximal end of the trachea was tied around the cannula and 1.0 mL of sterile PBS (Gibco, Grand Island, NY, USA) was instilled into the lungs and recovered by aspiration three separate times. The BAL fluid was centrifuged at 250 g to collect cells. The supernatant from the first pull was stored at \(-80^\circ\)C for later analysis of cytokines/chemokines. Cells from the 3 ml were resuspended in PBS, counted using a hemocytometer and then cytospun (Cytopro Cytocentrifuge (Wescor Inc. Logan, UT, USA) onto slides. The slides were stained with Hema 3 stain set (Fisher, Kalamazoo, MI). Later, cell differential counts were carried out on the slides with a minimum of 200 cells per slide counted for differential analysis per mouse.

**Lung Histology**

After whole lung lavage, the lung from each treatment group was tied with a suture thread via the trachea to the cannula. Once tied, the lung was slowly removed from the thoracic cavity. Then the lungs were slowly inflated with 1 mL of 10% formalin (Sigma, St. Louis, MO). The lungs were hung under a pressure of 15 cmH\(_2\)O for 24 hr while submerged in 10% formalin to obtain uniform lung inflation during fixation. Subsequently, the lung tissues were arranged in cassettes and send to a tissue processing facility where the lungs tissue were dehydrated and embedded in paraffin. 5 \( \mu M \) sections were made from lung tissue. The sections were stained for hematoxylin
and eosin (H&E) or utilized later for immunohistochemistry. Each slide was reviewed at scanning magnifications (×2, ×4, and ×10 objectives; Nikon Eclipse model E600 microscope). The slides were reviewed to assess the influx of inflammatory cells (mostly neutrophils).

**Immunohistochemistry**

Immunohistochemical staining of the lung tissue section was performed as previously reported (361) with slight modification. Formalin-fixed, paraffin-embedded sections of 5-µm-thick tissue were deparaffinized through Safeclear II™ tissue clearing agent (Fisher) and later rehydrated using a graded series of alcohol washes (100%, 95%, 80%, 50% ethanol). The slides were then rinsed 3 times in PBS. A heat-induced epitope retrieval method was performed for antigen unmasking. Briefly, slides were immersed in preheated antigen retrieval solution (DIVA Decloaker solution; Biocare Medical, Concord, CA) and steamed for 20 min at 95°C in a vegetable steamer. After heating, the slides were allowed to cool and then rinsed with PBS for 3 times. After washing, the slides were incubated with 0.1% Triton in PBS for another 10 min. The slides were then washed with PBS for 3 times for 5 min each. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 15 min. After being washed 3 times in PBS, slides were blocked with non-fat milk (5%) in PBS-tween (0.1%) for another 2 hr in a humidity chamber at RT. Slides were incubated overnight with primary antibody and respective isotype control in a humidity chamber: rabbit anti-MAA and rabbit-IgG (dilution 1:1000; Abcam, Inc., Cambridge, MA). After being washed, slides were incubated with the appropriate HRP conjugated goat-anti-rabbit IgG (dilution 1:1000; Jackson ImmunoResearch Laboratories, Inc., Grand Island, NY) secondary antibody in a humidity chamber. After 1 hr, slides were rinsed and developed with Chromogen substrate (IMMPACT DAB, Vector, Burlingame, CA) followed by counter staining with 1% Meyer's hematoxylin. Finally, the slides were dehydrated through a series of ethanols and fixed with Safeclear II™ tissue clearing agent.
Cytokine/Chemokine Assays

Murine, keratinocyte chemoattractant (KC; CXCL1) level was determined in BAL fluid according to manufacturer's instructions using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Lung Slice Protocol

Lung slices were prepared as previously described (88). Briefly, C57BL/6 mice, between 7 and 9 weeks old, were sacrificed by isoflurane overdose. The trachea was cannulated with an intravenous (IV) catheter tube with two input ports (20G Intima; Becton Dickinson) and secured with suture thread. Lungs were allowed to deflate after which a syringe filled with a warm (37 °C) solution of 2% agarose (type VII or VII-A: low gelling temperature; Invitrogen, Carlsbad, CA, USA) in Hanks Balanced Salt Solution (HBSS; pH 7.4) was slowly instilled into the lung until fully inflated. Immediately after agarose inflation, the lungs were washed with ice-cold HBSS, packed with ice and allowed to cool at 4 °C for 30–45 min. To ensure the complete gelling of the agarose, the lungs were further placed in cold HBSS solution for an additional 30 min–1 hr. A single lobe of lung was then removed and placed in the mounting block of the vibratome of the Electron Microscopy Sciences Tissue slicer (OTS 4500) to make lung sections. The lung lobe was sectioned into slices 150 μm thick. Sections of the lung were then transferred to wells of a 24-well plate and maintained with DMEM supplemented with 10% FBS, antibiotics, and antimycotics at 37 °C and 5% CO₂ for at least 5 days prior to treatment. Twenty-four hours prior to treatment, media was changed to serum free media. Lung slices were then treated with SPD-MAA for 24 hr. After 24 hr the supernatant was collected and stored at -80 °C for further chemokine analysis. The lung slice from each treatment were collected and homogenized in cell lysis buffer and the protein concentration (mg/mL) was measured using the Bradford assay (362). The chemokine level was normalized to the concentration of protein.
Real-time Quantitative RT-PCR (qRT-PCR)

RNA was isolated from the lung tissue using RNeasy mini kit (Qiagen, Valencia, CA) following manufacturer instructions as previously described (363, 364). After RNA isolation, the concentration and purity of RNA was determined by NanoDrop spectrophotometer and samples had A260/A280 ratio of 1.9–2.1. TaqMan reverse transcription kit (Applied Biosystems, Branchburg, NJ) was used to synthesize DNA from 100ng of template RNA purified from lung tissue. cDNA synthesis (RT-PCR) reactions contained the following reagents: 1 × TaqMan RT buffer, 5.5 nM MgCl₂, 500 µM of each dNTP, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl MultiScribe Reverse Transcriptase. Samples were incubated at 25 °C for 10 min, then 48 °C for 30 min, and 95 °C from 5 min in a thermocycler (MJ Mini; Bio-Rad, Hercules, CA). Real-time quantitative PCR was performed on the cDNA using the following reaction: 1× Taqman master mix and mouse CD204 primer (Applied Biosystems, Branchburg, NJ; Mm00446214_m1) and probe mix in 25-µl reactions in a 96-well plate in duplicate. The plate was placed in an ABI Prism 7500 Sequence detection system (Applied Biosystems). Reactions were carried out for 2 min at 50°C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data are reported as fold-change from control.

SRA ELISA

For measurement of SRA expression on whole lung tissue, the protein was isolated from the lung tissue. The lung tissue was rinsed in PBS to remove excess blood. The tissue was then homogenized in 500 µl of PBS at 4 °C. The homogenate was then centrifuged at 15,000 g for 10 mins. After centrifugation, the supernatant was collected and protein was measured using the Bradford assay. The supernatant was used to perform the sandwich ELISA according to manufacture’s instructions (LifeSpan Bioscience Seattle, WA).
Statistical Analyses

All data were analyzed using Graphpad Prism 5 (San Diego, CA, USA). Results represent mean ± standard error. One- and two-way ANOVA with Tukey post-hoc test and one-sample t-test were used to analyze data for statistical significance. $P < 0.05$ was accepted as statistically significant.

5.3 Results

SRA KO mice showed decreased lung cellularity following repetitive MAA instillation.

Repetitive instillation of SPD-MAA has been previously reported to increase cellular influx in lavage fluid in WT mice. When SRA KO mice were repeatedly instilled with SPD-MAA (50 µg/mL) for 3 weeks, however, a significant decrease ($p < 0.01$) in total lung lavage cells was observed when compared to WT mice (Fig. 17). No difference was observed in both WT and SRA KO mice instilled with saline or non-adducted SPD. These data suggest that SRA could be important in SPD-MAA mediated effects on lung cellularity. This result also suggests that SPD-MAA mediated increases in total BAL cellularity is SRA dependent.
Figure 17. Total cell count in BALF after 3 weeks of intranasal instillation of SPD-MAA.

Total cell count in BALF from WT and SRA KO mice after intranasal instillation of saline, SPD-MAA (50 µg/mL) and SPD (50 µg/mL) for 3 weeks. Values are presented as the mean ± SEM, n=6-10 mice/group.

**SRA KO mice showed decreased neutrophil influx following repetitive MAA instillation.**

Repeated instillation for 3 weeks of 50 µg/mL SPD-MAA resulted in an increased influx of neutrophils in lung when compared to the saline-instilled group (Fig. 18C). There were no changes in eosinophils (Figure 18A) or lymphocytes (Fig. 18B). When SRA KO mice were repeatedly instilled with SPD-MAA, significantly (p < 0.01) fewer neutrophils were observed when compared to SPD-MAA instilled WT mice (Fig. 18C). Likewise, the seemingly decrease in macrophages was seen in WT mice after MAA instillation compared to WT saline (Fig. 18D) is a result of the increased neutrophils represented in the total 200 cells counted for each treatment groups. Saline and non–adducted SPD instillation have no effect on the neutrophil or macrophages count in the BAL fluid. These results suggest that the decrease in total lung cellularity observed after SPD-MAA instillation in SRA KO mice was due to a decreased influx of neutrophils compared. These results suggest that SRA is important for MAA-mediated neutrophil recruitment.
Figure 18. Differential white blood cell counts in BALF collected after intranasal administration of SPD-MAA, SPD or saline.

Differential cell eosinophils (A), lymphocytes (B), neutrophils (C) and macrophages (D) count in BALF from WT and SRA KO mice after intranasal instillation of saline, SPD-MAA (50 µg/mL) and SPD (50 µg/mL) for 3 weeks. Values are presented as the mean ± SEM, n=6-10 mice/group.

MAA adduct-stimulated chemokine release is SRA-dependent in vitro.

To determine the role of SRA in MAA adduct-stimulated chemokine KC release, precision cut lung slices were made from both WT and SRA KO mice and later treated with media control, SPD-MAA (50 µg/mL) and non-adducted SPD (50 µg/mL) for 24 hr. KC levels in BAL samples from mice instilled for 1 day, 1 week, 2 weeks or 3 weeks instilled mice was also measured. No increase in KC release was observed over any of the time period in saline instilled WT mice (Fig.
19A). In the SPD-MAA instillation group there was a significant increase in KC release in the 1-week (p < 0.001) and 2 week (p < 0.05) mice when compared to the 1-day animals (Figure 19B). At 3 weeks, KC release returned to baseline levels (Fig. 19B). In SRA KO mice there was no significant increase in KC release over time in both saline-instilled (Fig. 19C) and SPD-MAA instilled mice (Figure 19D). In support of these observations, ex vivo treatment with SPD-MAA resulted in a significant increase (p < 0.0001) in KC release from lung slices made from WT mice when compared to control treatment (Fig. 3E). In contrast, a significant reduction (p < 0.0001) in KC release was observed from lung slices of SRA KO mice when compared with WT lung slices treated with MAA (Fig. 19E). No increase in KC release was observed over the time period of SPD instillation in WT mice (data not shown). These data show that SRA is required for MAA-stimulated chemokine release in an *in vitro* lung slice model.
Figure 19. Neutrophil chemokine KC (CXCL1) in BAL and from lung slices.

KC levels in BAL samples of WT mice (A and B) and SRA KO mice (C and D) instilled with saline or SPD-MAA: 50 µg/mL were measured over 1d, 1-3 weeks time. Lung slices from both WT and SRA KO mice were treated with SPD-MAA (100 µg/mL) for 24 hr (E). After 24 hr supernatant was collected and neutrophil chemokine KC release was measured. Values are presented as the mean ± SEM of n=3 independent experiments for lung slice and n=5-17 mice per group for BAL KC release.
Repeated exposures to MAA adduct increased whole lung SRA expression.

To determine the role of MAA adduct on lung SRA (CD204) expression, lung tissue from WT mice instilled with 50 µg/mL SPD-MAA for 3 weeks was used to measure SRA message and protein expression. Repeated MAA exposure resulted in significant increase (p < 0.05) in SRA message expression (almost two-fold)(Fig. 20A). Consistent with the message expression, a significant increase (p < 0.05) in SRA protein expression was also observed (Fig. 20B). No such increase in both SRA message and protein was seen in WT mice instilled with non-adducted SPD (Fig. 20A, 20B). These data may be important in explaining the increased inflammation in WT mice due to repeated exposure to MAA adduct.

Figure 20. mRNA and protein levels of SRA (CD204) in total lung tissue.

mRNA levels of SRA on whole lung tissue after 3-week saline, SPD-MAA (50 µg/mL) and SPD (50 µg/mL) instillation. Values are presented as the mean ± SEM, n=5 mice/group.
SRA KO mice display reduced lung inflammation following repeated MAA adduct instillation.

Our previous study shows that repetitive exposures to MAA adduct results in lung inflammation due to the influx of inflammatory cells influx within the peri-bronchiolar region of small airways (237). To determine the role of SRA on such inflammation, SRA KO mice were instilled with 50 µg/mL of SPD-MAA adduct for 3 weeks and later paraffin-embedded whole lungs were sectioned and stained for hematoxylin and eosin. In comparison to WT mice (Fig. 21B), microscopic examination revealed significantly fewer inflammatory cells within the peri-bronchiolar region of the small airways of SRA KO mice exposed to MAA adduct (Fig. 21E). No such change (influx of neutrophils) was observed in the lungs of mice instilled with either saline (Fig. 21A,D) or non-adducted SPD (Fig. 21C,F) for both strains of mice.
Figure 21. Lung inflammation in lung after 3 weeks intranasal instillation of SPD-MAA.

Both WT and SRA KO mice were treated intranasally with saline, SPD-MAA (50 µg/mL) and SPD (50 µg/mL) for 3 weeks. A representative 4-5 um thick section of H and E stained of one mouse per treatment group is shown (10 X magnification). WT Saline (A), SPD-MAA (B), SPD (C) and SRA KO Saline (D), SPD-MAA (E), SPD (F). Line scale represents approx. 100 µM. Arrow denotes the localization of inflammatory cells.

SRA KO mice display reduced immunoreactivity for MAA adduct after repeated exposure.

Previous studies have shown that SRA is important for MAA adduct binding in lung epithelial cells (237). To further investigate that the decreased MAA mediated effect in SRA KO mice could be due to decreased binding of MAA adduct in lung tissue after 3-week instillation, whole lung sections from both WT and SRA KO mice were stained for MAA adduct. Immunohistochemical staining for MAA adduct demonstrated increased immunoreactivity along the bronchial epithelial and columnar epithelial cells around the airways in WT mice (Fig. 22B).
No staining was observed in WT and SRA KO mice instilled with saline (Fig. 22A) or non-adducted SPD (Fig. 22C). No staining was observed with isotype control antibody (data not shown). In contrast to WT mice, a remarkable decrease in MAA adduct reactivity was observed in SRA KO mice (Fig. 22E). These results suggest that SRA is important for MAA binding in airways as no such staining was detected in KO mice. This could further explain the decreased response to MAA adduct in SRA KO mice.

Figure 22. Representative lung tissue sections immunohistochemically stained for SPD-MAA.

Immunohistochemical staining of SPD-MAA in lung airways of both WT and SRA KO mice treated intranasally with saline, SPD-MAA (50 µg/mL) and SPD (50 ug/mL) for 3 weeks. A representative 4-5 µm-thick section of one mouse per treatment group is shown (20 X magnification). WT Saline (A), SPD-MAA (B), SPD (C) and SRA KO Saline (D), SPD-MAA (E), SPD (F). Line scale represents approx. 100 µM. Arrow denotes the staining and binding of MAA adduct.
5.4 Discussion

In this study, we demonstrated that SRA plays an important role in regulating MAA-adduct induced lung inflammation and lung injury. Repetitive instillation of MAA adduct in mouse lung resulted in inflammation as a result of influx of neutrophils influx in the peri-bronchial region. Increased lung cellularity and increased influx of neutrophils were observed in the lungs of WT mice, but in SRA KO mice all of these effects caused by MAA adduct after repetitive instillation were significantly diminished. This diminished inflammation in SRA KO mice could be further explained by a decrease in MAA adduct binding to the airway cells. As a result, a decrease in chemokine KC release from the lung slices was observed. Similarly, no significant increase in KC release in BAL from SRA KO mice instilled with SPD-MAA at 1 week and 2 weeks was observed. This could explain the decreased neutrophil influx in the lung of the SRA KO mice after MAA adduct instillation. An increase in KC release in BAL from WT mice instilled for 1 week and 2 weeks suggests earlier KC release leading to the recruitment of the neutrophils in the airways at 3 weeks. Our study is the first one to show the importance of SRA in MAA-induced lung inflammation in mouse lung.

Previous studies show that macrophages scavenge chemically modified proteins such as formaldehyde-treated bovine serum albumin, maleylated albumin and malondialdehyde-modified and acetylated low-density lipoprotein by endocytosing these modified proteins via a receptor mediated mechanism (365). Highly reactive aldehydes formed as a result of smoking and drinking have the ability to modify lung proteins to produce a highly stable and immunogenic product, malondialdehyde-acetaldehyde (MAA) adduct (87, 231). In contrast to individual protein adducts formed by AA and MDA, which are unstable and dissociate rapidly, this MAA adduct is stable and can remain in the lung for a long time (366, 367). In mouse lung, surfactant proteins A (SPA) and D (SPD) are equally MAA-adducted when exposed to both ethanol and cigarette smoke (88). This suggests that surfactant proteins are ideal targets for MAA adduction in lung. The presence of this adduct in liver, lung and recently in the rheumatoid arthritis (RA) synovial
tissue could suggest its importance as a marker of inflammation (250). In lung, SRA expressed on bronchial epithelial cells is reported to bind MAA adduct and internalize them (237). In support of this result, we earlier reported that SRA was involved in MAA-adducted protein-stimulated, PKC/ε-mediated KC production in mouse airway epithelial cells (237). We show that MAA adduct binding to bronchial epithelial cells is blocked when pretreated with fucoidan, a ligand for scavenger receptor A (239). Additionally, a decreased antibody response to MAA-Alb in SRA KO mice was also reported (235). Because SRA is extensively expressed in macrophages, a similar result was also reported when macrophages from SRA KO mice were treated with MAA adduct. A decrease in the pro-inflammatory cytokines, TNF alpha and IL-6, was observed when compared to macrophages from WT mice (360). Willis et al. also reports that MAA-haptenated proteins are preferentially bound by scavenger receptors on macrophages, which internalize the ligands and shuttle them to lysosomes (368). Our result further supports these published results that SRA is one of major receptors involved in the effects mediated by MAA-modified proteins.

There is a similarity between the mechanism by which both oxidized LDL (ox-LDL) and modified MAA protein initiate toxicity (369). Both of these are recognized by scavenger receptors and Ox-LDL is associated with the pathogenesis of atherosclerosis (370). There are also various theories suggesting the specific feature of the ligand that make it suitable for the scavenger receptor. Reaction of amino groups of protein with short-chain α-hydroxy aldehydes may yield a moiety that is important for recognition by scavenger receptor A (371). Negative charges in the ligands could be another important feature for bonding reaction (372). Change in protein confirmation after binding with aldehydes could be another determinant in receptor binding (373). MAA adduct formed as a result of Schiff base reaction between 2 moles of malondialdehyde and 1 mole of acetaldehyde with lung protein, such as SPD, therefore, may fulfill the above-mentioned requirements, making it a ligand for SRA.

Our study also showed a difference in MAA staining in the lung after repeated intranasal instillation for 3 weeks in WT and SRA KO mice. Interestingly most of the positive staining for
MAA was seen in airway epithelial cells, the cells that first come into contact with MAA after intranasal instillation. Airway epithelial cells express SRA and take up MAA as previously reported (237). The remarkable finding of our study was that in SRA KO mice, significantly less MAA staining was observed. This further supports the observation that the diminished response to MAA in SRA KO mice is due to less uptake of MAA by airway epithelial cells. Our results also suggest that binding of MAA to airway epithelial cells is important to initiate the inflammatory responses to MAA.

Our study also demonstrated an increase in SRA expression after repetitive instillation of MAA adduct for 3 weeks. The increased inflammation in the lung of WT mice after MAA instillation could be due to increased binding as a result of increased expression of the SRA on the lung. This modulation of SRA expression in lung is important as it could modulate the inflammatory responses to MAA adduct. Chronic lung inflammation is one of the characteristic features of COPD, which is predicted to be the third leading cause of death worldwide by 2030 (374). Smoking is the primary risk factor for COPD (375) and about one-third of smokers are drinkers. This is why it is important to study the role of co-exposure of cigarette smoke and alcohol on lung inflammation. One of the pathways through which this co-exposure could induce lung injury is the formation of MAA adduct from reactive aldehydes. Our results suggest that SRA is important to initiate MAA-mediated inflammation in lung. Previous studies have also reported that genetic polymorphisms in macrophage scavenger receptor-1 gene (MSR1) could result in increased macrophage adhesion, receptor expression, and cell number (376). It has been also linked to COPD susceptibility (377).

**5.5 Conclusion**

In summary, this study demonstrates the functional role of SRA in MAA adduct-induced inflammation in lung. SRA is important for initiation of inflammation after instillation of MAA as absence of SRA is protective against MAA mediated lung inflammation and injury. Future
studies involving individuals with genetic polymorphisms in SRA would be important to better understand the role of SRA on susceptibility to lung inflammation in smokers and drinkers.
Collectively, alcohol abuse and cigarette smoking are known for causing hundreds of thousands of preventable deaths each year. In lung, several reactive aldehydes such as acetaldehyde, malondialdehyde and 4-HNE are generated as a result of drinking and smoking. High concentrations of these aldehydes lead to the formation of DNA and protein adducts. In this study, we showed that malondialdehyde can form DNA adduct (M1dG) in both bronchial epithelial cells and mouse lung, leading to the induction of DNA damage. But after treatment with the CYP2E1 inhibitor DADS, the formation of this adduct and DNA damage was reduced. In addition to this, our study also showed that a hybrid adduct (MAA), formed when reactive acetaldehyde and malondialdehyde covalently bind to proteins to form a stable hybrid adduct in lung, could initiate several inflammatory reactions in both in vitro macrophage and in vivo mouse models. Our study further confirmed that SRA is one of the major receptors for MAA adducted protein in macrophage and mouse models.

Individuals who abuse alcohol and smoke cigarettes are at risk for generating reactive aldehydes and ultimately adducts in lung, which may represent an early biomarker for adverse health outcomes. Thus, it would be beneficial to implement an easy to use technique for the detection of high levels of relative aldehydes and adducts in biological fluids. Such techniques would be helpful to identify individuals at risk for developing adduct-related inflammation and lung injury. The early detection of these aldehyde adducts could help in development of interventions to further prevent the progression of lung diseases. Aldehydes and MAA adducts in BAL fluid and exhaled breath condensate could be easily measured by liquid chromatography-tandem mass spectrometry to monitor the disease and its response to therapy. These biomarkers may help to identify smokers and drinkers who are at a greater risk of developing alcoholic lung disease and COPD. This would also provide a rationale for the evaluation of conventional
therapies and for the development of novel drugs. These markers could be of prognostic value for lung disease in smokers and drinkers. Importantly, adduct formation could be one of several potential mechanisms of how drinking alcohol and smoking cigarettes could increase the risk of DNA damage and inflammatory lung disease.

Our study also showed that CYP2E1 is important for the formation of reactive aldehydes. Inhibiting this enzyme would be beneficial to minimize the adverse effects of these adducts. Implementation of this strategy would play an important role for screening at risk individuals and targeting individuals at need for clinical testing and/or dietary supplementation. Genetic difference plays an important role in how an individual responds to aldehydes and their respective adducts because some people may have an increased risk of aldehyde-related disease, whereas others are protected by their genetic CYP2E1 profile. Detailed future studies concerning individual susceptibility to high aldehydes and adducts stratified by polymorphisms in CYP2E1 is necessary before recommending a particular dose of DADS.

DNA adduct formation is considered a critical step in the process of carcinogenesis. M1dG adduct formation could lead to DNA damage that is quite persistent, with a relatively long half-life of 12.5 days. Therefore it is of clinical importance to study how these DNA adducts induce mutations and chromosome abnormalities. M1dG could be established as a biomarker of oxidative stress and lipid peroxidation due to dietary habits and environmental exposures. This would help to predict risk for cancer development and tumor progression. Our study has tried to broaden knowledge about the importance of dietary supplementation in the prevention of oxidatively damaged DNA. To date, little is known about M1dG adduct formation and its effects on lung tumor progression, but no correlation has yet been established between them. Therefore, it is difficult to establish an association between exposure and disease because the process is complex and their real impact are unknown or not easily estimable due to many factors such as individual genetic profile, age, diet, lifestyle, health status, and mode of exposure. With this in mind, our preliminary findings could be useful to characterize the risk, at least in term of DNA
damage, for those individuals who abuse alcohol. Still, more detailed studies on these adducts, their receptors, and their signaling pathways are warranted in the future. We also need to analyze the levels of M1dG adduct, a biomarker of oxidative stress and lipid peroxidation, in a cohort of alcohol abusers. Furthermore, the relationship between alcohol exposure levels and M1dG adducts need to be evaluated. The dose of alcohol to which the workers are consuming and consequent DNA adduct formation will help in planning future preventive actions. These findings would be of clinical importance to develop effective preventive interventions.

Not all alcohol abusers who smoke cigarettes will develop symptoms of injury due to aldehyde adducts suggesting genetic factors may likely be important. Keeping this in consideration, polymorphisms in SRA may be important, as it is the major receptor for the MAA adduct. This will help to identify individuals at risk as well as suggest novel targets for drug treatments. Previously the role of SRA polymorphism in COPD susceptibility among smokers has been established, but the role of SRA in alcohol and cigarette smoke mediated lung effects have not. Therefore, it is necessary to investigate SRA genetic polymorphisms in disease susceptibility in such individuals. Our study highlighted the role of SRA on MAA-mediated lung inflammation and injury. The data from macrophage and mouse studies further confirmed that SRA is necessary for MAA-mediated effects. This opens future directions to further exploring the role of SRA in alcohol abusers who also smoke cigarettes. In addition, a future SRA genetics study may help to predict who will get lung disease or who will be protected from unfavorable outcomes among drinkers who also smoke cigarettes.

Modification of surfactant protein D (SPD) due to MAA adduction is another important mechanism that could be addressed in future studies. Surfactant protein plays an important role in innate defense as well as in inflammatory regulation within the lung. Deficiency in oligomerized SPD might contribute to the pathogenesis of a variety of human lung diseases. Multimeric SPD is anti-inflammatory because its hydrophobic N-terminus is hidden and thus not able to bind to its calreticulin/CD91 receptor complex and initiate pro-inflammatory responses. But chemical
modification of SPD can lead to disruption of its multimeric structure into trimers exposing the hydrophobic N-terminus. Such modification enhances SPD ability to bind to its calreticulin/CD91 receptor complex and initiate a pro-inflammatory response. This suggests that any modification in SPD structure is important for its role in inflammation. Also, depletion of SPD results in decreased clearance of microorganisms and exaggerated pro-inflammatory response in lung damage. Because MAA adduct is formed when acetaldehyde and malondialdehyde attack SPD protein, it is necessary to study whether such adduction will alter SPD structure, its receptor binding ability, and its biological functions. In addition to direct structure-function modifications, it should be determined whether MAA adduction of SPD results in decreasing free SPD levels in lung and if this could lead to significant changes in innate defense. Such findings will help to better understand the mechanism behind the observed reduced innate immunity of alcohol abusers who also smoke cigarettes.

In conclusion, our findings demonstrate that alcohol abuse and cigarette smoking are involved in the process of aldehyde adduct formation, such as M1dG and MAA in the airways and play an important role in airway inflammatory and DNA damage processes. Our study will help to establish links between aldehyde formation and lung disease, especially in a population of those with alcohol use disorders (AUDs) who also smoke cigarettes. This doctoral thesis gives a comprehensive overview about reactive aldehyde and their role in various lung pathologies. These findings provide fundamental data for further analysis in the clarification of the relevance of genetic variations in CYP2E1 and SRA genes in relation to aldehyde adduct-mediated lung injury. Hopefully, this will help to advance the development of nutritional strategies and health recommendations to address disease susceptibility through the use of a biomarker for early identification of alcohol abuse and cigarette smoking-related lung injury.
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