Intact β-Cryptoxanthin Prevention of Cigarette Smoke-Induced Inflammatory Lung Lesions Independent of Carotenoid Cleavage Enzymes and Mechanistic Understanding of Sirtuin 1 Activity

A thesis presented to the faculty of the Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy

By:

Rachel Anna Chiaverelli, M.S. / P.S.M.

In partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Tufts University

February 2018

Thesis Committee:

Xiang-Dong Wang, M.D. / Ph.D. (Advisor and Chair)

Dayong Wu, M.D. / Ph.D.

Irfan Rahman, Ph.D.

Virendar Kaushik, Ph.D.
Acknowledgements

I would like to take the time to acknowledge everyone who contributed to the successful completion of my doctoral thesis work.

First, and foremost, I would like to thank my research advisor, Dr. Xiang-Dong Wang. From my initial interview at Tufts University to the completion of my doctoral work, Dr. Wang has been nothing but supportive, providing his expertise and guidance in every aspect of my work. Most importantly, Dr. Wang’s excitement over research never goes away, and never goes unnoticed. Without his exceptional levels of motivation towards my research projects and research in general, I would have never been able to successfully complete my dissertation.

Thank you to my committee members, Dr. Dayong Wu, Dr. Irfan Rahman and Dr. Virendar Kaushik. Throughout my thesis work, you all provided me with great comments, questions and advice that were instrumental in the progression of my research. Your encouragement and direction drove my successful navigation of this doctoral work, even when unexpected outcomes resulted. Together, you all were extremely helpful, and I am so grateful for your support.

Thank you to the entire Nutrition and Cancer Biology laboratory for always providing a supportive environment for the entirety of my doctoral degree. Thank you to Dr. Chun Liu for, not only your extensive pathology work, but for the support system and friendship that you have given me during my time at Tufts. Connie Hu, thank you for always being available to teach any skill imaginable in the lab and providing your expert opinion of techniques. Dr. Junrui Cheng, Ji Ye Lim, and Jelena Mustra Rakic, thank you for your support, advice, and, most importantly, lifelong friendship we have established throughout completion of my degree.

Thank you to my academic advisor, Dr. Alice Lichtenstein for always being there to support my progress, whether through coursework, Gershoff symposiums or guidance for life outside the walls of the HNRCA. Thank you to Dr. Stanley N. Gershoff and the Stanley N. Gershoff Fellowship, and as well as the Provost Fellowship and James A. Sadowski Internship Fund, providing me with funding throughout my studies.

I would also like to thank Dr. Donald Smith, Jonathan Morrison, and the rest of the team in the Comparative Biology Unit for helping me to plan and execute my animal studies in the HNRCA. I could not have completed everything without everyone’s efforts.

Lastly, the endless encouragement and support from my family and friends throughout this entire process was, as we say it in biochemistry, the rate-limiting enzyme in this process. Without you, I would have never gotten this far, or successfully completed this degree. To my loving parents, Lucy and Tony, for always being accepting of my goals and encouraging me through difficult times. To my brother, Nick, and my goddaughter, Adrianna, for giving me the motivation to finish and be a role model to her success in the future. To my friends, for providing me with an outlet and supportive environment to keep focused and on track.
Abstract

Cigarette smoke (CS) inhalation remains a major public health problem. Chronic inflammation induced by CS is known to be the underlying mechanism for many diseases. Inflammation from CS-exposure has been shown to lead to bronchitis and emphysema, which together, chronically, lead to irreversible damage and chronic obstructive pulmonary disease (COPD). COPD remains a leading cause of hospitalization and is expected to become the third most common cause of death by 2020. Currently, therapeutic interventions for COPD only help with the symptoms of the disease, but no cure exits. Therefore, the need for the discovery of dietary preventative agents is necessary.

Several epidemiological studies, including the National Health and Nutrition Examination Survey (NHANES) and the Seven Cohorts Studies, have shown an increase in the plasma levels of carotenoids was associated with lower risk of chronic lung disease. Specifically, β-cryptoxanthin (BCX), a pro-vitamin A carotenoid abundantly found in butternut squash, pumpkin, citrus fruits, papaya, and sweet red peppers, was the only carotenoid for which intake was associated with a lower risk of lung disease, such as lung cancer, in current smokers. However, the protective effect of BCX against other lung diseases, such as emphysema as a result from CS-induced inflammation has not been investigated. Moreover, the biologically active effects of intact BCX, without the generation of vitamin A or apo-10’-carotenoids, via β-carotene-15,15’-oxygenase (BCO1) and β-carotene-9’,10’-oxygenase (BCO2), respectively, has not been investigated. BCO1/BCO2 polymorphism has been associated with alterations in human and animal carotenoid level status, consequently impacting the generation of vitamin A and apo-carotenoids generated by BCO1/BCO2, which possess strong, well-known biological activities. However, whether the presence of BCO1/BCO2 polymorphisms in the human population has implications in carotenoid metabolism and function remains unclear. In this thesis work, we investigated whether BCX possesses anti-inflammatory biological activity to prevent CS-induced inflammation and lung lesion development and the potential mechanism(s) involved, and whether this biological activity is independent of, or dependent on, carotenoid cleavage enzymes, BCO1/BCO2.

In the first part of this study, we examined the preventative effects of biologically active, intact BCX supplementation (20 mg/kg diet), against two weeks of CS-exposure in both wild type (WT) and BCO1/BCO2 double knock out (DKO, BCO1−/−/BCO2−/− which both BCO1 and BCO2 cleavage enzymes have been knocked out) mouse model, including males and females. We observed that BCX supplementation was able to significantly reduce CS-induced inflammation and emphysema in both DKO and WT mice in the same manner, as measured by inflammatory cell infiltration and the average distance between alveolated membranes (Lm) and hyperplastic bronchiolars, respectively, therefore no genotype effect was revealed. This response was associated with HPLC analysis of the liver, which demonstrated a significant accumulation of BCX in both mouse genotypes, with less accumulation in the WT mice. Moreover, as expected, WT mice showed a significant increase in retinol generation when fed BCX, whereas DKO did not show any change in retinol levels. Thus, indicating a protective effect of BCX due to the activities of intact BCX. Molecularly, the pathological findings of intact
BCX biological activities were consistent with reduced the gene expression of inflammatory cytokines (IL6 and TNFα) and matrix metalloproteinases (MMP-2 and MMP-9), related to inflammation and inflammatory lung lesion development. No sex differences were observed. This part of the thesis provides the first in vivo evidence that intact BCX possesses its own anti-inflammatory activity, and has protective effects against CS-induced lung lesions, independent of the generation of vitamin A or apo-10’-carotenoids.

In the second part of this thesis work, we aimed to examine the underlying mechanism by which inflammatory lung lesions develop in response to CS. Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylase, known for its anti-inflammatory and anti-aging activity, has been introduced as a potential target of CS due to its sensitivity to redox status and inflammation. Moreover, decreased levels of SIRT1 protein have been found in patients suffering from COPD, however much controversy exists over the implications of SIRT1 protein levels versus its activity, therefore SIRT1 protein levels alone cannot be used to reflect its deacetylase activity. To address this question, we conducted a CS-exposure study using both male and female mice which have been genetically modified so that they do not have whole body SIRT1 enzymatic activity (Sirt1⁺/⁺), or have partial SIRT1 enzymatic activity (Sirt1⁺/⁻), compared to wild type (WT) mice with fully functioning SIRT1 enzymatic activity (Sirt1⁺/⁺). Following CS-exposure for six weeks, Sirt1⁺/⁻ and Sirt1⁺/⁻ mice had a significant inflammation response compared to Sirt1⁺/⁺ mice not exposed to CS. To our surprise, Sirt1⁺/⁻ mice exposed to CS produced a weak inflammatory response, but did have an exacerbation of emphysema, compared to Sirt1⁺/⁺ and Sirt1⁺/⁻ mice. We investigated this novel finding molecularly and discovered a significant decrease in transcription factors related to cell cycle progression and a significant increase in markers of cellular senescence in the lungs of both Sirt1⁺/⁻ and Sirt1⁺/⁻ mice. Specifically, we discovered a CS-induced decrease in transcription factor, p53, which is related to the regulation of cell cycle progression. Consistent with these findings, we revealed a decrease in cell cycle progression (cyclin-dependent kinase 1, CDK1) and an increase in biomarkers for cell arrest/cellular senescence (p21 and p16), which is likely the underlying mechanism for emphysema development, independent of inflammation. We did not observe sex differences. The second part of this thesis presents evidence that the whole-body loss of SIRT1 enzymatic activity results in the exacerbation CS-induced emphysema with much less of an inflammatory response, as compared with WT mice. This study lays the groundwork for further investigation of SIRT1 enzymatic activity’s role in CS-induced cell arrest and senescence as the underlying mechanism for emphysema development.

Taken together, using two genetically modified mouse models, this thesis work demonstrates that, first, intact BCX, independent of carotenoid cleavage enzymes is a strong anti-inflammatory agent against CS-induced inflammation and lung lesions without sex differences; and, secondly, SIRT1 enzymatic activity plays a major role in the underlying mechanism of emphysema induction due to cell arrest and senescence, without sex differences.
Table of Contents

Intact β-Cryptoxanthin Prevention of Cigarette Smoke-Induced Inflammatory Lung Lesions Independent of Carotenoid Cleavage Enzymes and Mechanistic Understanding of Sirtuin 1 Activity------------------------------------ i

Acknowledgements ---------------------------------- ii

Abstract ------------------------------------------- iii

Tables ----------------------------------------------- 1

Figures ----------------------------------------------- 2

Introduction ------------------------------------------- 4

Significance ------------------------------------------- 8

Specific Aims ----------------------------------------- 9

Specific Aim 1 --------------------------------------- 10

Specific Aim 2 --------------------------------------- 11

Literature Cited -------------------------------------- 13

Figure 1: Cigarette Smoke (CS)-Induced Inflammation and Protease Secretion------- 22

Figure 2: Specific Aim 1 Hypothesis ------------------------------------------- 23

Figure 3: Specific Aim 2 Hypothesis ------------------------------------------- 24

Review of the Literature--------------------------------- 25

Cigarette Smoke, Cigarette Smoke-Induced Inflammation and Inflammatory Lung Lesions ----------------------------------------------- 26

Carotenoids----------------------------------------------- 30
β-Cryptoxanthin

Sirtuin 1, Sirtuin 1 Role in Inflammation, Emphysema, and Chronic Obstructive Pulmonary Disease

Summary

Literature Cited

Tables and Figures

Figure 1: Six Common Carotenoids

Table 1: β-Cryptoxanthin (BCX) Food Sources

Figure 2: BCX Structure and Cleavage

Figure 3: Sirtuin 1 Response to Oxidative Stress

Manuscripts

Chapter I

Intact β-cryptoxanthin prevents lung inflammation independent of carotenoid cleavage enzymes

Abstract

Introduction

Materials and Methods

Results

Discussion

Author Contributions
Figure 1.7: β-Cryptoxanthin (BCX) treatment reduced inflammation, increased Sirtuin 1 (SIRT1) protein and decreased phosphorylation of Protein kinase B (pAkt/Akt) in lipopolysaccharide (LPS)-stimulated BEAS-2B cells. 107

Figure 1.8: β-Cryptoxanthin (BCX) metabolite, 3-OH-β-apo-10’-carotenal (3OH-BA10C) protected against inflammation, increased Sirtuin 1 (SIRT1) protein and decreased phosphorylation of Protein kinase B (pAkt/Akt) in lipopolysaccharide (LPS)-stimulated BEAS-2B cells. 108

Chapter 2: Mechanistic Role of Sirtuin 1 Enzymatic Activity in Cigarette Smoke-Induced Emphysema. 110

Abstract. 111

Introduction. 113

Materials and Methods. 116

Results. 119

Discussion. 121

Acknowledgments. 125

Literature Cited. 126

Tables and Figures. 132

Table 2.1: Mouse Study Outcomes. 133

Table 2.2: Pathological Report of Emphysema Cases and Inflammation Grade. 134
Figure 2.1: mRNA Expression of Inflammatory Markers including Interleukin-6 (IL-6), Tumor necrosis factor (TNF)-α, and Interleukin-10 (IL-10)  

Figure 2.2: mRNA Expression of Matrix Metalloproteinases (MMPs), MMP-12, MMP-2, MMP-9  

Figure 2.3: mRNA Expression of Nicotinamide Phosphoribosyltransferase (NAMPT)  

Figure 2.4: Potential mechanisms by which CS-exposure impacts NAMPT, leading to emphysema in Sirt1/y/y mice  

Figure 2.5: mRNA Expression of Markers of Cell Cycle Progression and Cell Senescence  

Figure 2.6: Conclusion  

Summary and Discussion  

Limitations and Strengths  

Future Directions
### Tables

<table>
<thead>
<tr>
<th>Section</th>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lit. Rev.</td>
<td>1</td>
<td>BCX Food Sources</td>
<td>61</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.1</td>
<td>$BCO1^{+/+}/BCO2^{+/+}$ Double Knockout and Wild Type Mouse Study Outcomes</td>
<td>100</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.1</td>
<td>Mouse Study Outcomes</td>
<td>133</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.2</td>
<td>Pathological Report of Emphysema Cases and Inflammation Grade</td>
<td>134</td>
</tr>
</tbody>
</table>
### Figures

<table>
<thead>
<tr>
<th>Section</th>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intro</td>
<td>1</td>
<td>Cigarette Smoke-Induced Inflammation and Protease Secretion</td>
<td>22</td>
</tr>
<tr>
<td>Intro</td>
<td>2</td>
<td>Specific Aim 1 Hypothesis</td>
<td>23</td>
</tr>
<tr>
<td>Intro</td>
<td>3</td>
<td>Specific Aim 2 Hypothesis</td>
<td>24</td>
</tr>
<tr>
<td>Lit. Rev.</td>
<td>1</td>
<td>Six Common Carotenoids</td>
<td>60</td>
</tr>
<tr>
<td>Lit. Rev.</td>
<td>2</td>
<td>BCX Structure and Cleavage</td>
<td>62</td>
</tr>
<tr>
<td>Lit. Rev.</td>
<td>3</td>
<td>Sirtuin 1 Response to Oxidative Stress</td>
<td>63</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.1</td>
<td>Hepatic BCX, Retinol and Retinyl Palmitate as Detected by HPLC</td>
<td>101</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.2</td>
<td>CS-induced inflammation and changes to pulmonary structure in lungs of $BCO1^{-/-}/BCO2^{-/-}$ Double KO mice</td>
<td>102</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.3</td>
<td>BCX feeding significantly prevented inflammation in the lung of $BCO1^{-/-}/BCO2^{-/-}$ and WT mice exposed to CS</td>
<td>103</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.4</td>
<td>BCX feeding significantly prevented inflammation in the lung of $BCO1^{-/-}/BCO2^{-/-}$ mice exposed to CS</td>
<td>104</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.5</td>
<td>BCX supplementation significantly inhibited CS-exposed induced lung structural pathology changes in $BCO1^{-/-}/BCO2^{-/-}$ and WT mice</td>
<td>105</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.6</td>
<td>BCX supplementation significantly inhibited CS-exposed induced matrix metalloproteinase expression in the lung of $BCO1^{-/-}/BCO2^{-/-}$ DKO mice $BCO2^{-/-}$ mice</td>
<td>106</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.7</td>
<td>BCX treatment reduced inflammation, increased SIRT1 protein and decreased phosphorylation of Akt in LPS-stimulated BEAS-2B cells</td>
<td>107</td>
</tr>
<tr>
<td>Ch.1</td>
<td>1.8</td>
<td>BCX metabolite, 3OH-BA10C protected against inflammation, increased SIRT1 protein and decreased phosphorylation of Akt in LPS-stimulated BEAS-2B cells</td>
<td>108</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.1</td>
<td>mRNA Expression of Inflammatory Markers</td>
<td>135</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.2</td>
<td>mRNA Expression of Matrix Metalloproteinases</td>
<td>136</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.3</td>
<td>mRNA Expression of Nicotinamide Phosphoribosyltransferase (NAMPT)</td>
<td>137</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.4</td>
<td>Potential mechanisms by which CS-exposure impacts NAMPT, leading to emphysema in Sirt1y/y mice</td>
<td>138</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.5</td>
<td>mRNA Expression of Marker of Cell Cycle Progression and Cell Senescence</td>
<td>139</td>
</tr>
<tr>
<td>Ch.2</td>
<td>2.6</td>
<td>Conclusion</td>
<td>140</td>
</tr>
</tbody>
</table>
Introduction

Despite a number of public health campaigns, cigarette smoking remains a major public health problem in the United States and worldwide (1–3). Furthermore, rates of smoking have increased in women, making their consumption equivalent with that of men (2). Cigarette smoke (CS) is a major oxidative burden composed of a mixture of toxic substances (4–6), resulting in diseased-state pathophysiological consequences, especially increased inflammation (7–9).

Chronic inflammation is known to be the underlying mechanism for the development of respiratory diseases due to the oxidative burden of CS (9–11). Inflammation from CS-exposure has been associated with the development of inflammatory lung lesions which lead to emphysema, which, chronically, can lead to irreversible damage and chronic obstructive pulmonary disease (COPD) (7,8,10,12).

In brief, the current understanding of CS-induced inflammation and emphysema is that the oxidative burden of CS causes the secretion of chemokines from the epithelial cell lining of the alveolar membrane in the lung, and this release stimulates the recruitment of inflammatory cells, predominantly neutrophils and macrophages to the lung. The infiltration of these inflammatory cells, work with the adaptive immune system to respond to the oxidative burden of CS, and in turn, a cascade of inflammatory responses occurs stimulating further recruitment of inflammatory cells, and provoking the release of protease, which to cleave matrix proteins within the lung, thereby creating alveolar airspace enlargement and destruction, which eventually develops into emphysema (Figure 1) (12,13). The primary class of proteases involved in this process are matrix metalloproteinases (MMPs) (14–18). Evidence also suggests that MMPs
themselves cause further drive inflammation during their respective proteolytic cleavage functions from classical matrix substrates, and in some cases, non-matrix related substrates, thereby exacerbating the deleterious effects of CS on the pulmonary membranes (19).

Chronically, the emphysemic effects of damage to the lung infrastructure leads to the development of permanent damage and chronic obstructive pulmonary disease (COPD) (20–22). COPD pathogenesis is very complicated and involves a complex interrelated network of immunopathological processes (21,23). The complex nature of both emphysema and the progression to COPD makes the proper and timely diagnosis and efficacious development of therapeutics a daunting task, and no cure currently exists (24). The currently available pharmacological therapies on the market only help to alleviate the exacerbations seen in patients suffering with emphysema and COPD, and these include inhaled bronchodilators, antibiotics, corticosteroids, oxygen, methylxanthines, mucolytic agents, and adjunct therapies (25).

Alongside smoking cessation, these therapies can only help the patient cope with their symptoms, however epidemiological studies have demonstrated the powerful role that dietary patterns, namely increasing consumption of fruits and vegetables rich in carotenoids, resulting in increased plasma levels of carotenoids which have been associated with improved pulmonary function and a decreased risk for lung diseases, such as chronic obstructive pulmonary disease and lung cancer, in smokers (26,27). Previous clinical studies examining β-carotene, however, have proved to be major failures (28,29). The Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) and the Carotene and Retinol Efficacy Trial (CARET) revealed interactions between β-
carotene and cigarette smoke exposure, proved ineffective, and even harmful at high
doses, demonstrating the pro-carcinogenic effects of a supra-physiological dose β-
carotene (30).

Despite the clinical failures of β-carotene, evidence suggests another provitamin A carotenoid, β-cryptoxanthin (BCX), an oxygenated carotenoid (xanthophyll),
abundantly found in butternut squash, red sweet peppers, tangerines, oranges, peaches,
and pumpkins, possesses unique anti-inflammatory and anti-carcinogenic functions (31–
33). National Health and Nutrition Examination Survey III (NHANESIII) and other
epidemiologic studies, including seven large well-implemented cohorts, that have shown
that a high serum level of BCX found in fruits and vegetables is associated with a lower
risk of lung cancer death in current smokers, independent of intakes of other nutrients
such as vitamin C, folate, and other carotenoids including α-carotene, β-carotene, lutein,
lycopene and zeaxanthin (27,34,35). Preclinically, our group, and others, have
demonstrated the in vitro and in vivo potential for BCX to function as both an effective
preventative anti-tumorigenic dietary agent, as well as having anti-inflammatory
capabilities (36–39).

When metabolized BCX is cleaved by two carotene β-oxygenases. β-Carotene-
15,15′-oxygenase (BCO1) cleaves carotenoids, such as β-carotene and BCX at the 15,15′
double bond, which is critical for retinoid production and homeostasis (40,41). β-
carotene-9′,10′-oxygenase (BCO2), cleaves both β-carotene and BCX at the 9′,10′ double
bond to generate both apo-10′-carotenoids and 3-hydroxyl (3-OH) apo-10′-carotenoids
(42–44). Due to this metabolism of BCX, in conjunction with epidemiological findings
and the presence of single nucleotide polymorphisms (SNPs) in BCO1 and BCO2 in the
population (45,46), the question of the biological activity potential of intact BCX has been raised.

In addition to preclinical evidence of BCX influence on classical indicators of inflammation, work from our laboratory has demonstrated BCX supplementation in mice with expression of both \textit{BCO1/BCO2} to have the ability to influence Sirtuin 1 (SIRT1), a class Ia mammalian sirtuin localized in the nucleus, cytoplasm and mitochondria linked to anti-aging properties (47,48). SIRT1 functions as a histone deacetylase (HDAC) and its enzymatic activity is dependent on nicotinamide adenine dinucleotide (NAD)$^+$ recycling, therefore, SIRT1 is a very sensitive target to changes in redox due to endogenous sources such as CS (49–51). Moreover, decreased plasma SIRT1 levels have been identified in COPD patients (52). It is important to note, however, that SIRT1 protein and/or enzymatic activity level do come with some controversial results from the laboratory findings. Because of its ability to deacetylate many substrates, SIRT1 has had implications in the protection from various diseases, as well as the promotion of disease. Thus, SIRT1 and its close relationship to CS-induced inflammation, and its clinical relevance to patients suffering from COPD, must be better understood in a preclinical model which specifically examines the whole-body enzymatic activity of SIRT1 in relation to CS-exposure.
**Significance**

There is a vital need to: 1) Continue to research the deleterious effects of CS-exposure and its consequential induction of emphysema to further promote the public health initiatives to stop the prevalence of smoking at the population level; 2) Develop effective dietary preventative interventions to stop the development of emphysema and/or the progression of emphysema to irreversible damage in COPD; 3) Provide relevant preclinical animal models in order to better understand the underlying mechanisms by which these complicated disease states occur; 4) To improve the current knowledge within the scientific community to improve proper identification of biomarkers and diagnosis, to create targeted dietary strategies, effective therapeutics and treatment alternatives. This thesis project set out to tackle the four pillars mentioned above, utilizing two novel, genetically modified mouse models to make a noteworthy contribution to the scientific community to better understanding the pathogenesis of CS-induced inflammation and inflammatory lung lesions, and provide outstanding evidence that an efficacious dietary intervention is plausible in ameliorating CS-induced inflammation and lung lesions.
Specific Aims
The overall objective of this thesis work is to mechanistically understand the biochemical, molecular and inflammatory consequences of CS-induced inflammation and inflammatory lung lesions, first, to identify BCX as a biologically active, intact compound to prevent CS-induced inflammation and inflammatory lung lesions, and then to determine the mechanistic role that SIRT1 enzymatic activity plays in CS-induced inflammation response and emphysema case development in two separate genetically modified mouse models.
Specific Aim 1

Determine the biological action of BCX, independent of its cleavage enzymes, in CS-smoke induced immunological responses and lesions in lungs (Figure 2) We hypothesize that intact BCX possess biological activity that works to prevent CS-induced lesions. In the absence of carotenoid cleavage enzymes BCO1 [β-carotene 15,15′-oxygenase], for vitamin A formation, and BCO2 [β-carotene 9’,10’-oxygenase], for apo-10’-carotenoids formation, intact BCX molecule can confer preventative biological action against CS-induced lung inflammation and pathological damage. Results showed that the BCX (20 mg/kg diet) supplementation, without altering vitamin A levels, prevented smoke-induced lung inflammation in absence of BCO1/BCO2. We used male and female $BCO1^{-/-}/BCO2^{-/-}$ knockout mice exposed to tobacco smoke-exposure with and without administration of BCX, compared to mice not exposed to tobacco smoke with and without BCX feeding, to determine the biological action of intact BCX in the prevention of lung lesions following cigarette smoke-exposure and identify any sex differences within groups. We also examined the WT counterparts, with and without BCX feeding, for comparison of BCX accumulation in the liver. We observed an accumulation of BCX, without its conversion into vitamin A and apo-carotenoids. Thus, demonstrating that intact BCX is able to deliver protection against CS-induced inflammation and pathological damage in the absence of BCO1/BCO2 enzymes in the $BCO1^{-/-}/BCO2^{-/-}$ knockout mouse model. We did not observe significant sex differences in terms of BCX accumulation, vitamin A formation, response to CS-exposure and response to BCX intervention.
Specific Aim 2

Elucidate the impact that ablation of SIRT1 enzymatic activity has on tobacco-smoke exposure-associated inflammation and lung lesion development, via a complex network of molecule pathways (Figure 3) We hypothesize that 1) SIRT1 is a molecular target of tobacco smoke-exposure; 2) Insufficient SIRT1 activity will affect the body’s immune response to CS-exposure; 3) Mice with insufficient SIRT1 (Sirt1<sup>y/y</sup>) enzymatic activity will have increased tobacco smoke-induced damage due to a weakened immunological defense system. SIRT1, an NAD<sup>+</sup>-dependent deacetylase, encompasses a wide range of controversial roles in the body, including immune defense against environmental toxins, such as tobacco smoke. Furthermore, preliminary data showed that the ablation of SIRT1 activity resulted emphysema development in time-dependent manner in homozygous Sirt1<sup>y/y</sup> mice. We will utilize male and female mice completely lacking SIRT1 enzymatic activity (Sirt1<sup>y/y</sup>), partial SIRT1 activity (Sirt1<sup>+/y</sup>), and full (wild-type, WT) SIRT1 activity (Sirt1<sup>++/+</sup>), exposed to short-term tobacco smoke, to examine SIRT1’s role in tobacco smoke-induced inflammatory lung lesions due to its function in the body’s immunological response. We expected that the insufficient or lack of SIRT1 activity would enhance smoke-induced inflammation and emphysema development, as compared with their corresponding wild type mice, but surprisingly, this study revealed that Sirt1<sup>y/y</sup> mice did not produced a CS-induced inflammatory response, measured at both the pathological and molecular levels. Sirt1<sup>y/y</sup> mice did, however, have a significant increase in the amount of emphysema cases due to CS-exposure, compared to that of WT mice exposed to CS. Molecularly, this increase in emphysema was found to be, surprisingly, independent of increased expression in MMPs. Therefore, in an attempt to better understand the underlying molecular explanation for these unexpected findings,
we examined the expression of p53, a transcription factor related to cell cycle progression and found it was significantly decreased. Moreover, gene expression revealed that biomarkers of cellular senescence (p21, p16) in the lungs of Sirt1^{+/y} and Sirt1^{+/y} mice were significantly increased and cell cycle progression was decrease (CDK1). Taken together, SIRT1 enzymatic activity’s role in CS-exposure may have a more direct role in the development of emphysema, due to an increase in cell arrest and senescence, independent of an inflammatory response.
Literature Cited


43. Mein JR, Dolnikowski GG, Ernst H, Russell RM, Wang X-D. Enzymatic formation of apo-carotenoids from the xanthophyll carotenoids lutein, zeaxanthin and β-


Cigarette smoke-induced inflammation and protease secretion: Cigarette smoke increases inflammation via stimulation of various inflammatory cells, in parallel, this increase in inflammation leads to protease secretion which, in combination, further drives inflammation and increases alveolar airspace enlargement and destruction, which is observed in emphysema.
Specific Aim 1 Hypothesis: We hypothesized intact BCX possesses biological activity, independent of cleavage enzymes, prevents CS-induced inflammatory lung lesions, and that gender differences could be a contributing factor for BCX metabolism and inflammatory lung lesion development.
**Figure 3: Specific Aim 2 Hypothesis**

**Specific Aim 2 Hypothesis:** We hypothesized SIRT1 is a molecular target of tobacco smoke-exposure (CS) and, therefore, ablation SIRT1 activity will affect the body’s inflammatory response to CS-exposure. Gender differences could be a contributing factor inflammatory lung lesion development.
Cigarette Smoke, Cigarette Smoke-Induced Inflammation and Inflammatory Lung Lesions
Despite major public health initiatives to combat cigarette smoking in the U.S. and worldwide, smoking remains a major problem (1). The rate of women who smoke is on the rise, and now is equivalent to that of men (2). Smoking is a major contributor to a wide range of various diseases, most closely related being lung diseases including emphysema, bronchitis, COPD, and lung cancer (3).

CS induces a large oxidative burden to the host organism, and therefore many different physiological consequences arise (4,5). CS does not discriminate against its targets within the body, however the central site of damage is the pulmonary airways (6–8). Cigarette smoke (CS) also drives oxidative damage while decreasing the efficacy of the body’s endogenous antioxidant response (9–11). Reports have shown that this ability for smoke to drive redox imbalance induces an inflammatory response, characterized by an abundance of alveolar macrophages, neutrophils, and T and B lymphocytes, which is a central driver of lung lesion severity (12–14). CS has been linked to autoimmunity and autoimmune diseases such as lupus by interrupting processes leading to autoimmunity, i.e. provoking oxidative stress, which dysregulates DNA methylation, upregulating immune genes, thereby leading to an autoimmune response within the body (15). Furthermore, the immune system becomes increasingly susceptible to pathogen introduction, and even with smoking cessation in COPD patients, there is a continued inflammatory response, indicating a potential, self-perpetuating mechanism within the body and a potential role of the adaptive immune system (16). In addition to inflammation, CS possess the ability to trigger endothelial dysfunction and alteration of the lung microenvironment, leading to structural and pathological changes in the lungs, leading to lesions, and, chronically, COPD (12,14,15,17).
CS smoke not only impairs proper gas exchange between airways, it increased the secretion of proteases from the epithelial lining of the pulmonary membranes, and these proteases work to cleave proteins which support the membrane structures of the alveolar membranes within the lung (18–24). Once this occurs, emphysema may arise, and long-term this destruction becomes permanent and irreversible damage (25). In parallel, when exposed to CS, the membranes within the lung try to protect themselves from the large oxidative burden by secreting mucus. This mucus contains a variety of immune cells, as well as inflammatory cytokines, and other signaling proteins. When mucus hypersecretion occurs from a consistent insult of CS, bronchitis develops (26).

Emphysema and bronchitis, in combination, long-term, lead to COPD (12,27,28).

Due to the lack of mucus hypersecretion in mouse models of CS-exposed lung disease, the damage related to the inflammatory response is due to inflammatory lung lesions, which lead to emphysema development and COPD (29–31). The current understanding of CS-induced inflammation and emphysema is that the oxidative burden of CS causes the secretion of chemokines from the epithelial cell lining of the alveolar membrane in the lung, and this release stimulates the recruitment of inflammatory cells, predominantly neutrophils and macrophages to the lung. The infiltration of these inflammatory cells, work with the adaptive immune system to respond to the oxidative burden of CS, and in turn, a cascade of inflammatory responses occurs stimulating further recruitment of inflammatory cells, and provoking the release of protease, which to cleave matrix proteins within the lung, thereby creating alveolar airspace enlargement and destruction, which eventually develops into emphysema (Figure 1) (32,33). The primary
class of proteases involved in this process are matrix metalloproteinases (MMPs) (34–38). Evidence also suggests that MMPs themselves cause further drive inflammation during their respective proteolytic cleavage functions from classical matrix substrates, and in some cases, non-matrix related substrates, thereby exacerbating the deleterious effects of CS on the pulmonary membranes (24). This damage, long term, lead to COPD, permanent and irreversible damage to the lung, that severely dampers the lifestyle and capabilities of inflicted individuals (39).

Today, CS-exposure kills more than 480,000 every year, and the average rate of death of COPD due to CS is on the rise, and is forecasted to continue to increase by the year 2020 (1,40). Currently, no effective treatments exist for emphysema, bronchitis, or COPD (17,41). The only therapeutics currently on the market do not treat the disease, rather they help to improve the symptoms of the diseases (5). Thus, the need for a preventative intervention is key to aid in halting the onset and development of emphysema and bronchitis, as well as slowing or inhibiting the onset of COPD.

Improvement in dietary habits provides and easily modifiable and inexpensive lifestyle change in comparison to the current therapeutics available on the market. Epidemiological studies including large cohorts of individuals, over long periods of time have shown strong and significant associations with dietary lifestyle modifications and/or habits and their relationship to either health or disease. Specifically, with the identification of biologically active metabolites in foods and the developments in metabolomics, we now know that foods, especially fruits and vegetables are abundant sources of carotenoids possessing active metabolites that may have the ability to confer powerful health properties on the individuals (42–44).
Carotenoids
Carotenoids are a family consisting of over 700 fat-soluble pigments found in fruits and vegetables (FAV) (42,45,46). Six classes of carotenoids are commonly studied due to their biological activity and functions. These include: α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene and zeaxanthin (43). Carotenoids can be separated into two groups, those that do not form vitamin A, non-provitamin A, and those that form vitamin A, provitamin A carotenoids. Non-provitamin A carotenoids include lutein, lycopene, and zeaxanthin (Figure 1) (47).

Carotenoids have been associated with lower risk of various diseases. The biological activities of carotenoids include antioxidant and inflammatory modulating properties (48). Some antioxidant activities of carotenoids include quenching free radicals, reducing damage from free reactive oxygen species and inhibiting lipid peroxidation (43,49). Because there are currently no known preventative dietary agents or treatments for CS-induced inflammatory lung lesions or COPD that have proved effective (10,11,41,50). Recommendations to increase consumption of fruits and vegetables in order to sustain a healthful life and reduce individuals’ risk for a number of chronic diseases have been implemented by health professionals and nutritional guidelines for a substantial number of years (42,51–54). In a recent, controlled clinical study in males and females, researchers identified plasma levels of carotenoids, specifically β-cryptoxanthin (BCX), lutein and zeaxanthin, were positively associated with daily consumption of fruits and vegetables (42). Furthermore, with respect to CS-induced lung disease, epidemiologic studies have shown that increased consumption of fruits and vegetables rich in carotenoids and, thus, increased serum levels of carotenoids
have been associated with a decreased development of COPD in smokers, higher levels of FEV1, and lowered long-term COPD mortality (55).
β-Cryptoxanthin
\(\beta\)-cryptoxanthin (BCX) is a common oxygenated carotenoid (xanthophyll) that can be found in colorful FAV, predominantly butternut squash, persimmons, hot chili peppers, red sweet peppers, tangerines, oranges, and other foods (45,56) (Table 1).

BCX has been reported to be a reliable biomarker of FAV intake, as higher levels of BCX can be accurately detected and quantified in the plasma of humans following FAV consumption (42). Reports have shown that BCX functions as an antioxidant (55,57). Importantly, higher intake of the dietary, (BCX), abundantly found in has been associated with a lower risk of a number of certain chronic diseases including arthritis, osteoporosis, nonalcoholic fatty liver disease, esophageal cancer, head and neck cancer, prostate cancer, breast cancer, lung cancer and COPD (42,44,49,55,58–65). Specifically, a study showed higher levels of BCX, an oxygenated carotenoid (xanthophyll), with provitamin A activity, was not only positively associated with pulmonary function, but also plasma levels of BCX were increased in individuals who have greater cigarette smoke exposure, indicating a potential physiological response to increased oxidative burden (66). Additionally, recent data from the National Health and Nutrition Examination Survey III and other epidemiologic studies showed that high levels of serum BCX, not \(\beta\)-carotene, is associated with a lower risk of lung cancer death in current smokers, and smokers had lower circulating levels of BCX than non-smokers (67). Our lab has found physiologically-relevant levels of BCX to confer protective activity against pulmonary damage both \textit{in vitro} and \textit{in vivo} (68–70) and that BCX supplementation was effective in reducing the carcinogen-induced lung tumor multiplicity and nicotine-promoted emphysema, without a significant alteration of vitamin A (retinol and retinyl palmitate) concentration in both serum and lungs of the mice. Interestingly, BCX feeding
was effective in inducing sirt1 in mice, also without alteration of retinoid levels. This has raised a question as to whether the potential protective activity of BCX is due to the intact molecule or metabolites.

BCX’s pro-vitamin A activity occurs following cleavage by β-Carotene-15,15'-oxygenase (BCO1), which cleaves BCX at the 15,15’ double bond, which is critical for vitamin A production and homeostasis (45,71) (Figure 2). BCX also is cleaved by β-carotene-9’,10’-oxygenase (BCO2), which cleaves BCX at the 9’,10’ double bond to generate both apo-10’-carotenoids and 3-hydroxyl (3-OH) apo-10’-carotenoids (72,73). Although both vitamin A and apo-10’-carotenoids are biologically active by BCO1 and BCO2 cleavage, data suggest that intact BCX may be responsible for the protective biological functions observed in previous studies.

Recent reports have identified the presence of BCO1/BCO2 SNPs in humans, which could have relevant implications in the absorption, metabolism, and function of BCX concerning disease (57). The genetic variants of the BCO1 gene are prevalent in humans and other mammals and BCO1/BCO2 single nucleotide polymorphism (SNP) has been associated with alterations in the status of human and animal carotenoid levels (2). These studies raised an important question of whether the effect of BCX on various cellular functions and signaling pathways is a result of the direct actions of intact carotenoids or their enzymatic cleavage metabolites: vitamin A and apo-10’-carotenoids. Additionally, these studies suggest that differential expression of cleavage enzymes could then lead to alteration of storage of carotenoids in the hepatocytes and stellate cells of the liver (3). Altered storage of carotenoids could pose further downstream consequences on the actual bioavailability and function of the carotenoid itself, or vitamin A for that
matter, thereby having an altered biological function (2). Studies have also recently reported controversial results regarding gender and BCX absorption, as well as function to prevent CS-driven lung lesions and disease (42,44,55–57). In Japanese women, a common SNP in BCO1 gene, rs6564851, was identified and has implications in the circulating β-carotene (BC) levels (74). This study found a positive correlation with BCX intake and circulating BC levels in women GG homozygotes, compared with those carrying the T allele. This study is not the first of its kind, however, a genome-wide association study (GWAS) previously demonstrated the same result (75). Moreover, another GWAS study revealed a SNP in the BCO2 gene was associated with increased levels of IL-18 (76). Thus, suggesting a role that BCO2 may be playing in inflammation, although the underlying mechanism by which BCO2 and IL-18 interact is unknown.

Our group has demonstrated that BCX itself acts as a ligand for RAR transcription activation (77) and both BCX and its cleavage metabolite, 3-OH-β-apo-10’-carotenonal at comparable doses can attenuate LPS-induced inflammatory responses (78), and the inhibition of a specific nicotine receptor (α7-nAChR)/PI3K/AKT pathway contributes to suppression of cancer cell motility and angiogenesis by BCX itself (70). We provided intervention experimental evidence that BCX at both dietary and supplemental doses significantly decreased cigarette smoke-induced pre-cancerous lesions (69,77) and carcinogen-induced lung tumor multiplicity and nicotine-promoted emphysema, without a significant alteration of vitamin A (retinol and retinyl palmitate) concentration in both serum and lungs of the mice while the AIN-93M semi-purified diet contained sufficient vitamin A (70).
In this thesis work, we examined the effects of dietary BCX feeding intervention against cigarette smoke (CS)-induced lung inflammation in the absence of both BCO1 and BCO2 in mice [6]. We observed that BCX supplementation significantly decreased the amount of both neutrophil and macrophage infiltration, as well as the mRNA expression of IL6 and TNFα in the lung tissue of CS-exposed BCO1/BCO2 KO mice, with no sex differences, as compared to mice with CS-exposure alone. BCX supplementation also significantly decreased both the percentage of bronchiolar membranes with hyperplastic epithelium and the average distance between alveolated surfaces (Lm), accompanied with the down-regulation of mRNA levels of matrix metalloproteinases (MMP9 and MMP2) in the CS-exposed mice receiving BCX, compared to mice without BCX supplementation. Furthermore, BCX supplementation resulted in a significant accumulation of BCX in BCO1/BCO2 KO mice, but similar concentrations of retinol and retinyl ester, as compared with the non-supplemented mice. Therefore, in combination with other studies, this evidence clearly shows intact BCX as anti-inflammatory/anti-carcinogenic agent, independent of carotenoid cleavage enzymes, prevents inflammation-related chronic diseases.
Sirtuin 1, Sirtuin 1 Role in Inflammation, Emphysema, and Chronic Obstructive Pulmonary Disease
Sirtuin 1 (SIRT1) is a class Ia mammalian sirtuin localized in the nucleus, cytoplasm and mitochondria (13). SIRT1 functions as a histone deacetylase (HDAC) and its enzymatic activity is dependent on nicotinamide adenine dinucleotide (NAD\(^+\)). The hydrolysis of NAD\(^+\) is coupled with SIRT1 activity to transfer the acetyl group from the substrate to produce nicotinamide (NAM) and \(O\)-acetyl-ADP ribose, thus NAD\(^+\) serves as the rate limiting substrate for SIRT1 activity (79,80). Due to the dependence on NAD\(^+\), SIRT1 activity is especially sensitive to changes in the redox state of the cell (13,81,82). HDACs such as SIRT1 work to maintain the balance between acetylation and deacetylation of histones, thereby regulating downstream transcription and translation of protein products (13,81–83). If this balance is interrupted by endogenous toxins, such as CS-exposure, there are transcriptional consequences that could lead to disease.

CS-exposure causes oxidative stress, driving the development of inflammatory lung lesions, which occurs and results in a combination of activation of nuclear factor-kappa B (NF-\(\kappa\)B), impaired anti-protease defenses, DNA damage, autophagy, cellular senescence, autoantibody generation, and impaired histone deacetylases (13,16,84–89) (Figure 3). SIRT1, a nicotinamide NAD+-dependent histone deacetylase, has diverse roles in a broad range of physiological functions and has many implications in CS-driven inflammation inflammatory lung lesions and eventually COPD initiation and advancement (13,68,83,84,87). Due to the dependence on NAD\(^+\), SIRT1 is susceptible to changes in redox related bioenergetics. Moreover, decreased plasma SIRT1 levels have been identified in COPD patients (90). Environmental oxidative stress, such as CS-exposure, can induce redox modifications, thus impacting SIRT1 enzymatic activity and protein levels (86,91). Previous research has shown that decreased SIRT1 levels have
been observed in endothelial cells, macrophages, and neutrophils from bronchoalveolar lavage (BAL) samples extracted from the lungs of cigarette smokers (83,86,92).

Although previous research has shown a relationship between CS-exposure, SIRT1, lung lesions, and COPD, the underlying mechanism by which the enzymatic activity of SIRT1 impacts CS driven lung lesions via the immune response is not fully understood and represents a 1) vital avenue for better understanding of the development and progression of CS-induced inflammatory lung lesions and 2) potential molecular target for the prevention of this pulmonary damage. Moreover, work has found a potential link between SIRT1 and an autoimmune response to CS in COPD (89). Presently, no evidence has been shown that lack of SIRT1 enzymatic activity in vivo leads to an altered immunological response with CS-exposure, yet CS-exposure still produces alveolar airspace enlargement and bronchiolar hyperplasia. The relationship between enzymatic activity of SIRT1 and the inflammatory response, however remains unclear. Prior molecular evidence has shown the inflammatory consequences are due to SIRT1’s impact on NF-κB p65 activation via RELA acetylation (93,94), interaction with activating protein-1 (AP1) (95,96), downstream impact on extracellular matrix protein and adhesion molecule expression in the lung (97,98), and then impact nicotinamide phosphoribosyltransferase (NAMPT) expression and have downstream consequences on the CLOCK-BMAL axis (99–103).
Summary
Based on this review of the literature, there are significant gaps in the understanding of 1) CS-induced inflammation and inflammatory lung lesions, and the underlying mechanisms by which both of these pathophysiological processes occur, 2) Necessary potential for the discovery of biologically active dietary components that can prevent CS-induced inflammation and inflammatory lung lesions, 3) The role of SIRT1 enzymatic activity in CS-exposure, and its respective contribution to the inflammatory response associated with emphysema development, which chronically leads to COPD, and 4) the presence or absence of any sex differences. These research gaps led to the well-thought out development and execution of this thesis work. This thesis addresses all of the present gaps in research to the best of our knowledge, and also presents data for the development of future studies to further explore these research areas.

In part one of this thesis, we investigate the actions of intact BCX, independent of carotenoid cleavage enzymes, against CS-induced inflammation and inflammatory lung lesions. Then in part two, we determine the role that SIRT1 enzymatic activity plays in CS-induced inflammation and emphysema development. In both parts, we extensively measure the pathology and molecular markers of both lung in order to determine pulmonary inflammation and lung lesions, which contribute to emphysema. By addressing these pathophysiological changes in response to CS-smoke, we are able to assist in mending the present research gaps identified in the literature. The combination of parts one and two of this thesis work provide vital data and findings which both contribute to the current scientific literature and understand of CS-induce inflammatory and lung diseases, as well as provide insights for the future development of both preclinical and clinical studies.
Literature Cited


47. Carotenoids [Internet]. Linus Pauling Institute. 2014 [cited 2017 Nov 24].
Available from: http://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals/carotenoids

levels of inflammation, oxidative stress and NAD+ are linked to differences in plasma

carotenoid concentrations associated with a lower prevalence of the metabolic syndrome

50. Barnes PJ. Cellular and molecular mechanisms of chronic obstructive pulmonary

Fruit and vegetable intake and mortality from ischaemic heart disease: results from the
European Prospective Investigation into Cancer and Nutrition (EPIC)-Heart study. Eur
Heart J. 2011 May;32(10):1235–43.

potentially modifiable risk factors associated with myocardial infarction in 52 countries
(the INTERHEART study): case-control study. Lancet Lond Engl. 2004 Sep
11;364(9438):937–52.

and incidence of type 2 diabetes mellitus: systematic review and meta-analysis. BMJ.
2010 Aug 18;341:c4229.


Tables and Figures
**Figure 1: Six Common Carotenoids**

*Six common carotenoids: 1) Provitamin A carotenoids: a) β-carotene, b) α-carotene, c) β-cryptoxanthin; 2) Non-provitamin A carotenoids: a) lutein, b) zeaxanthin, c) lycopene*
Table 1: β-Cryptoxanthin (BCX) Food Sources

<table>
<thead>
<tr>
<th>Food</th>
<th>β-Cryptoxanthin (μg/100 g of food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butternut squash</td>
<td>3471</td>
</tr>
<tr>
<td>Persimmons</td>
<td>1447</td>
</tr>
<tr>
<td>Hubbard squash</td>
<td>1119</td>
</tr>
<tr>
<td>Hot chili peppers</td>
<td>1103</td>
</tr>
<tr>
<td>Tangerines (canned; raw)</td>
<td>775; 407</td>
</tr>
<tr>
<td>Papaya</td>
<td>589</td>
</tr>
<tr>
<td>Sweet red peppers</td>
<td>490</td>
</tr>
</tbody>
</table>

*Data from the USDA/ARS National Nutrient Database For Standard Reference, Release 27 (2014).*

Modified from Burri, et al, 2016

**BCX food sources:** BCX can be found in many colorful fruits and vegetables. Included in this table are some food sources containing the highest levels of BCX.
Figure 2: BCX Structure and Cleavage

**Vitamin A (β-apo-15-carotenoids)**

- β-carotene 15, 15’- oxygenase (BCO1)
- β-carotene 9’, 10’- oxygenase (BCO2)
- β-carotene 9’, 10’- oxygenase (BCO2)

**β-apo-10’-carotenoids**

**BCX Structure and Cleavage:** BCX can be cleaved by β-carotene 15, 15’-oxygenase (BCO1) at the 15,15’ double bond to form vitamin A (β-apo-15-carotenoids), or by β-carotene 9’,10’-oxygenase (BCO2) to form β-apo-10’-carotenoids.
Sirtuin 1 response to oxidative stress: Previous studies have shown SIRT1 decrease in response to oxidative stress (including CS), which impacts SIRT1 deacetylation activity, and consequently has implications in biological functions.
Manuscripts
Chapter I
Intact β-cryptoxanthin prevents lung inflammation independent of carotenoid cleavage enzymes
Rachel A. Chiaverelli¹², Chun Liu¹, Kangquan Hu¹, Jonathan R. Mein¹, Johannes von Lintig³, and Xiang-Dong Wang¹²*

¹Nutrition and Cancer Biology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA
²Tufts University Friedman School of Nutrition Science and Policy, Biochemical and Molecular Nutrition Boston, MA, USA 02111
³Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio, USA.

*Corresponding Authors:
Xiang-Dong Wang, MD, PhD
Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University
711 Washington Street, Boston, MA 02111
Phone: 617-556-3130 or 3144 Fax: 617-556-3344 or 3224
Email: xiang-dong.wang@tufts.edu

Abbreviations: 3OH-BA10C, 3-OH-β-apo-10’-carotenal ; BCO1, β-carotene-15,15’-oxygenase ; BCO2, β-carotene-9’,10’-oxygenase ; BCX, β-cryptoxanthin ; CS, cigarette smoke ; ERK 1/2, extracellular receptor activated kinases ; H&E, hematoxylin and eosin ; HO-1, heme oxygenase-1 ; IKK-β, IkappaB kinase-β ; IL-1β, interleukin-1β ; IL-6, interleukin-6 ; JNK, c-jun-N-terminal kinase ; LPS, lipopolysaccharide ; MAP, mitogen-activated protein ; TNF-α, tumor necrosis factor-α

Key words: Carotenoid metabolite, lung inflammation, cigarette smoke exposure, carotenoid cleavage enzymes
Abstract

Higher dietary β-cryptoxanthin (BCX) intake is associated with a lower risk of lung cancer in smokers. β-Carotene-15,15’-oxygenase (BCO1) and β-carotene-9’,10’-oxygenase (BCO2) cleave BCX, producing vitamin A and apo-10’-carotenoids. BCO1/BCO2 polymorphism has been associated with alterations in human and animal carotenoid level status. We investigated intact BCX protective effects against cigarette smoke (CS) induced-lung inflammation, independent of BCO1/BCO2. BCO1−/−/BCO2−/− double knockout mice (DKO) and wild type (WT) mice with full expression of BCO1/BCO2, fed BCX (20 mg/kg diet), exposed to CS, resulted in significantly decreased neutrophil and macrophage infiltration, percentage of bronchiolar membranes with hyperplastic epithelium, and average distance between alveolated airspaces (Lm) in lung tissue, with no genotype or sex differences, compared to mice with CS-exposure alone. Furthermore, we found that the inflammatory lung lesion development was associated with decreased IL6 and TNFα and matrix metalloproteinases (MMP9 and MMP2) mRNA expression, respectively, in lung tissue, again without sex differences. By HPLC analysis, in WT mice, hepatic BCX was detected with a significant increase in hepatic retinol without change in retinyl ester concentration with BCX feeding. In DKO mice, BCX feeding significantly accumulated hepatic BCX with no change in retinol or retinyl ester concentration, compared with control diet-fed mice. In vitro, BCX and BCX metabolite, 3-OH-β-apo-10’-carotenal (3OH-BA10C), pretreatment inhibited lipopolysaccharide (LPS)-induced mRNA expression of IL-6 and TNF-α and pAKT dose-dependently (1 to 4 µM) in human bronchial epithelial cell line, BEAS-2B, at comparable doses. This study demonstrates that BCX, either intact, independent of
carotenoid cleavage enzymes, is an anti-inflammatory agent, preventing CS-induced lung inflammation and lesions.
Introduction

Epidemiologic studies have shown that dietary intake patterns with increased consumption of fruits and vegetables rich in carotenoids, resulting in increased serum levels of carotenoids, has been associated with improved pulmonary function and a decreased risk for lung diseases, such as chronic obstructive pulmonary disease and lung cancer, in smokers (1,2). However, the identification of effective individual carotenoids, namely β-carotene, has not been successful. We have examined the potential interactions between β-carotene and cigarette smoke exposure, which proved ineffective, and even harmful at high doses, in both the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) and the Carotene and Retinol Efficacy Trial (CARET) (3,4). In contrast to β-carotene, we recently have been interested in whether β-cryptoxanthin (BCX), an oxygenated carotenoid (xanthophyll) with provitamin A activity, abundantly found in butternut squash, red sweet peppers, tangerines, oranges, peaches, and pumpkins, possesses unique anti-inflammatory and anti-carcinogenic functions. Previously, we have demonstrated that BCX inhibits the growth of immortalized human bronchia epithelial cells and non-small cell lung cancer cells and up-regulates retinoic acid receptor expression (5). Recently, we provided evidence that an experimental intervention with BCX, at both dietary and supplemental doses, significantly decreased cigarette smoke-induced pre-cancerous lesions and lung cancer in vivo (6,7). Additionally, we found that cigarette smoke exposure lowered plasma and lung tissue levels of BCX. This finding is consistent with recent data from the National Health and Nutrition Examination Survey III and
other epidemiologic studies, including seven large well-implemented cohorts, that have shown that a high serum level of BCX found in fruits and vegetables is associated with a lower risk of lung cancer death in current smokers, independent of intakes of other nutrients such as vitamin C, folate, and other carotenoids including β-carotene, β-carotene, lutein, lycopene and zeaxanthin (2,8,9). However, the biologic activities and underlying molecular mechanisms by which BCX protects the lung are poorly understood.

β-Carotene-15,15′-oxygenase (BCO1) cleaves carotenoids, such as β-carotene and BCX at the 15,15′ double bond, which is critical for retinoid production and homeostasis (10,11). Vitamin A and its derivatives, such as retinoic acid, serve critical functions in cellular proliferation and differentiation, immunity, and chronic lung disease (12). Indeed, we have shown that retinoic acid inhibited tumor formation in the lungs of tobacco smoke carcinogen-induced A/J mice (13). We recently demonstrated that BCX supplementation was effective in reducing the carcinogen-induced lung tumor multiplicity and nicotine-promoted emphysema, without a significant alteration of vitamin A, quantified as retinol and retinyl palmitate concentration in the serum, lung, and liver of mice while administered the AIN-93M semi-purified diet contained sufficient vitamin A (6,14). In addition to BCO1 cleavage, our team and others have demonstrated that a second enzyme, β-carotene-9′,10′-oxygenase (BCO2), cleaves both β-carotene and BCX at the 9′,10′ double bond to generate both apo-10′-carotenoids and 3-hydroxyl (3-OH) apo-10′-carotenoids (15–17). These studies raised an important question of whether carotenoids, such as BCX, which impact various
cellular functions and signaling pathways is a result of the direct actions of intact carotenoids or their metabolites. In the case of BCX, metabolites generated from cleavage include vitamin A and apo-10’-carotenoids (18,19). Moreover, genetic variants of the BCO1 gene are prevalent in humans and other mammals and a BCO2 polymorphism has been associated with alterations in the status of human and animal carotenoid levels (20,21). In humans, the prevalence of single nucleotide polymorphisms (SNPs) of BCO1 and BCO2 have been identified (20,22,23), and that disruption of BCO1 and/or BCO2 have implications in the cleavage of BCX and generation of BCX metabolites (24,25).

Furthermore, since β-carotene, a vitamin A precursor, did not provide any protection in the same A/J mouse model (26), and we have previously shown that BCX (7), not β-carotene (3), inhibited smoke-induced inflammatory lesions in the ferret, these data suggest that the preventive effect of BCX is likely due to the activity of intact BCX itself, rather than biologically generated vitamin A. We have also demonstrated apo-10’-lycopenoic acid, a BCO2 metabolite, induces Nrf2-mediated expression of phase II detoxification and antioxidant enzymes in human bronchial epithelial cells and inhibits the growth of lung cancer cells in vitro and suppresses lung carcinogenesis in A/J mice in vivo (5). Apo-carotenoid metabolites may have important biological roles different than their parent compound (27). The biological significance of carotenoid metabolite generation, especially the differential effects of small and large quantities of oxidative metabolites, has been reviewed (18). Despite well-documented research on the biological activities of BCO1 metabolites, retinoids (28), no studies have
investigated the potential biological activity of specific BCO2 cleavage product, 3-OH-β-apo-10’-carotenal (3OH-BA10C).

In the current study, we examined the effects of dietary BCX feeding intervention against cigarette smoke (CS)-exposure in mice in the absence of BCO1 and BCO2 cleavage enzymes, as well as in their corresponding WT littermates, with fully functioning BCO1 and BCO2. We then compared and confirmed the potential biological activity of BCX to its biologically active BCO2 metabolite, 3OH-BA10C, in vitro in a lung cell culture model.
Materials and Methods

1.1 Animal, diet and CS-exposure

Male and female $BCO1^{-/-}/BCO2^{-/-}$ double knock out (DKO) mice, established by Palczewski, et al (29) and wild type (WT) mice were randomly divided into groups: 1) Control; 2) + 20 mg/kg diet BCX; 3) CS-Exposure; 4) CS-Exposure + 20 mg/kg diet BCX. At 25 weeks of age, mice were fed BCX (>99% purity, BASF, Ludwigshafen, Germany), directly mixed with AIN-93M semi-purified diet (20 mg/kg diet), as previously described (6). At 27 weeks of age, mice received whole body, mainstream CS-exposure (Research Cigarettes, Type 3R4F) (7) for two weeks. CS-exposure was measured by urinary cotinine (30), by ELISA (ABNOVA, Taipei, Taiwan). This study was approved by the Animal Care and Use Committee at the HNRCA at Tufts University.

1.2 Pathology

The right upper lobe of each lung was inflated and fixed by gentle intratracheal infusion of 10 % neutral buffered formalin at 15 cm of H2O pressure for 2 minutes and then immersed in fresh 10 % neutral buffered formalin for a period of 72 hours. The samples were subsequently processed and embedded in paraffin, and serially sectioned. Four μm sections were stained with hematoxylin (H) and eosin (E) for histopathological examinations. Hyperplasia of bronchioles epithelium were examined for 20 fields at a magnification of x 100 (0.63 cm2) for each animal and expressed as % of bronchiolars with hyperplastic epithelium (the number of bronchiolars with hyperplastic epithelium divided by the total number of bronchiolars examined). To evaluate lung inflammation, macrophages and neutrophils were
quantified in the alveoli for 10 fields at a magnification of x 400 (1.59 mm²) for each animal, and the number of macrophages and neutrophils was expressed as cells/mm². The quantification of airspace enlargement was determined by the mean linear intercept (Lm) in micrometers. The measurement of Lm was performed by using a 100×100 μm grid that was randomly positioned over each field in the lung. The total length of each line of the grid divided by the number of alveolar intercepts yielded the average distance between alveolated surfaces, or the Lm. For each animal, 10 fields at a magnification of x 200 were examined. The sections were blindly examined by independent investigators by light microscopy [ZEISS Microscopy, PixeLINK USB 2.0 (PL-B623CU) Camera, PixeLINK µScope Microscopy Software].

1.3 High performance liquid chromatography (HPLC)

Liver samples of mice were prepared as previously described (31). Samples were reconstituted with 100 μL of a 2:1 ethanol:methyl-tert butyl ether solution. A gradient reverse-phase HPLC system consisting of a Waters 2695 separation module and a Waters 2998 photodiode array detector was used for the detection of BCX, retinol, retinyl palmitate and retinoic acid. BCX, retinol, retinyl palmitate and retinoic acid were analyzed on a reverse-phase C18 column (4.6 × 250 mm, 5 μm) (Vydac 201TP54, Grace Discovery Sciences, Inc., Bannockburn, IL) with a flow rate of 1.00 mL/min, and quantified relative to internal standards by determining the peak areas against known amounts of standards.

1.4 Cell culture and reagents

All-trans BCX (>99% purity, BASF, Ludwigshafen, Germany) and 3-OH-β-apo-10’-carotenal (3OH-BA10C, >99% purity, BASF, Ludwigshafen, Germany) were applied
to human bronchial epithelial cells, BEAS-2B, (ATCC, Manassas, VA) on plates pre-coated with bovine serum albumen/collagen/fibronectin with serum-free LHC-9 medium. Cells were pre-treated with BCX or 3OH-BA10C for 24 hours, and were stimulated with 10 ng/ml lipopolysaccharide (LPS), an established endotoxin (32), overnight.

1.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TriPure Isolation Reagent (Roche Applied Science). cDNA was prepared using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and an automated thermal cycler PTC-200 (MJ Research, Bio-Rad Laboratories, Hercules, CA). qRT-PCR was carried out using Fast Start Universal SYBR Green Master (ROX) (Roche, Indianapolis, IN). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

1.6 Protein isolation and Western blotting

Sample proteins were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). The following primary antibodies: phosphorylated-Akt (Ser473), total Akt, and total IkBa (Cell Signaling) and Sirt1, HO-1 (Santa Cruz) were used. Protein was detected by horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) and visualized by a SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL).

1.7 Statistical Analysis

Measurements are expressed as the mean ± the SEM, unless otherwise indicated. Comparisons across multiple groups were conducted by one-way ANOVA with Tukey Honestly Significant Difference (HSD). Differences between two groups were
analyzed by Student t test. Analyses were performed using GraphPad Prism (Version 7.01).
Results

1. Body weight remained unchanged among all groups in both genotypes, urinary cotinine significantly increased with CS-exposure

   Final body weight among all groups remained unchanged at week 29 (Table 1.1). Mouse weight was monitored weekly throughout the experiment, and the change in body weight (Final-initial body weight) among all groups, regardless of smoke exposure or dietary supplementation was not statistically significant (Data not shown). Urinary cotinine significantly increased in mice exposed to CS \((p<0.001\), Table 1.1). We observed a small amount of urinary cotinine in mice with no CS-exposure. Because these levels are very low in comparison to mice with CS-exposure, we believe this to be an endogenous metabolite \((33)\).

2. Hepatic vitamin A levels only increased in WT mice fed BCX, but did not differ between \(BCO1^{-/-}/BCO2^{-/-}\) DKO mice fed BCX and control diets, and smoke exposure decreased hepatic BCX levels only in WT mice, and hepatic retinol levels only in \(BCO1^{-/-}/BCO2^{-/-}\) DKO and WT mice

   BCX supplementation resulted in a significant accumulation of BCX \((30-43 \text{ nmol BCX/g liver})\) in the livers of DKO mice, but was non-detectable in non-supplemented mice. BCX accumulated in the liver of WT mice, but to a lesser extent \((1.7-10.2 \text{ nmol BCX/g liver})\) than in DKO mice (Figure 1.1). Interestingly, BCX significantly decreased in the liver of WT mice exposed to CS \((p<0.01)\). This is consistent to our previous findings in ferrets exposed to smoke and fed BCX. Similar concentrations of hepatic retinol and retinyl palmitate were detected in DKO mice with or without BCX supplementation; however, mice receiving smoke exposure showed a significantly
decreased level of hepatic retinol ($p<0.02$). WT mice fed BCX did have a significant increase in retinol levels ($p<0.05$). WT mice exposed to CS also had a decrease in hepatic retinol, consistent with the DKO mice. Hepatic retinoic acid was non-detectable among all groups (Figure 1.1). CS-exposed $BCO1^{-/-}/BCO2^{-/-}$ DKO mice fed BCX had significantly decreased inflammation in the lung.

3. Inflammatory cell infiltration of the lung significantly increased in DKO and WT mice exposed to CS, and BCX significantly decreased inflammation.

Two-weeks of CS-exposure significantly increased alveolar infiltration of inflammatory cells in the lung (Fig. 1.2D), as compared to mice without CS-exposure ($p<0.001$, Fig. 1.2A). Specifically, CS-exposure significantly increased both neutrophil ($p<0.001$, Fig. 1.3A) and macrophage ($p<0.001$, Fig. 1.3B) accumulation in the lung. However, mice exposed to CS and fed BCX had significantly decreased levels of inflammatory cell infiltration in the mouse lung ($p<0.001$, Fig. 1.3A and 1.3B). As indicated in Figure 1.3A and 1.3B, WT mice had the same inflammatory response. We chose to investigate the $BCO1^{-/-}/BCO2^{-/-}$ DKO mice at the molecular level because of our interest specifically in intact BCX. We found BCX supplementation decreased CS-exposed mRNA expression of TNFα ($p<0.001$, Fig. 1.4A) and IL-6 ($p<0.01$, Fig. 1.4B) in the lung, compared to mice exposed to CS without BCX supplementation. A wide variety of inflammatory cells produce TNF-α, namely alveolar macrophages and neutrophils, in the response to CS, and this production can stimulate the release of IL-6 (34).

4. CS-exposed $BCO1^{-/-}/BCO2^{-/-}$ DKO and WT mice fed BCX had a significant improvement in lung structural pathology.
Two weeks of CS-exposure induced significant alveolar airspace enlargement (Fig. 1.2B) and increased the number of bronchiolars with hyperplastic epithelium (Fig. 1.2C) in the mouse lung, in comparison to mice without CS-exposure \((p<0.001\), Fig. 1.2A). BCX supplementation significantly decreased the CS-exposure driven percentage of bronchiolar membranes with hyperplastic epithelium \((p<0.001\), Fig. 1.5A) and the average distance between alveolated airspaces (Lm) \((p<0.001\), Fig. 1.5B), a common quantitative measure of emphysema (35). We observed the same pathological response in both WT and \(BCO1^{-/-}/BCO2^{-/-}\) DKO mice, as indicated in Figure 1.5A and 1.5B. We chose to investigate the \(BCO1^{-/-}/BCO2^{-/-}\) DKO mice at the molecular level because of our interest specifically in intact BCX. We found that these pathological changes in response in BCX supplementation and CS-exposure were accompanied, molecularly, with the down-regulation of mRNA expression of matrix metalloproteinases, MMP-2 \((p<0.03\), Fig. 1.6A) and MMP-9 \((p<0.02\), Fig. 1.6B), compared to mice without BCX supplementation. MMP-2 and MMP-9 levels are both associated with CS-exposure destruction of extracellular matrix proteins in the lung (36).

5. BCX and BCX metabolite, 3OH-BA10C pretreatment inhibited lipopolysaccharide (LPS)-induced inflammatory responses and AKT phosphorylation in BEAS-2B cells at comparable doses.

BCX inhibited the LPS-stimulated expression of IL-6, TNF-\(\alpha\) and MCP-1 at mRNA in BEAS-2B cells in a dose-dependent manner (0.5 to 4 \(\mu\)M) (Fig. 1.7A). 3OH-BA10C also inhibited the LPS-stimulated expression of IL-6, TNF-\(\alpha\) and MCP-
I at mRNA in BEAS-2B cells in a dose-dependent manner (0.5 to 4μM) (Fig. 1.8A). The suppressing effects of BCX and 3OH-BA10C on IL-6 expression was associated with increased the expression of SIRT1 protein levels (Fig. 1.7B and Fig. 1.8B, respectively) and reduced AKT phosphorylation (Fig. 1.7C and Fig. 1.7C, respectively) in a dose-dependent manner, at comparable doses (0.5 to 4μM). Only BCX, not 3OH-BA10C, was able to up-regulate protein levels of IkB-α (Fig. 1.7D), negative regulator of NFκB, in a dose-dependent matter, without affecting Erk1/2 MAPKs (data not shown).
Discussion

To investigate the biological actions of intact BCX as its own molecule, we utilized a mouse model lacking both BCO1 and BCO2 carotenoid cleavage enzymes, as compared to their WT littermates, fully expression BCO1 and BCO2. We studied WT mice which fully express BCO1 and BCO2 in order to confirm the effectiveness of our DKO mouse model to study intact BCX and to address the biological activity of intact BCX, in addition to cleaved BCX. BCX dietary supplementation, at a physiological dose equivalent to daily human consumption of approximately 1.7 mg of BCX, which is physiologically attainable by consuming 3-5 raw tangerines daily (37), can prevent the damage induced by CS-exposure in male and female BCO1−/−BCO2−/− mice and in male and female WT mice, with no significant sex differences. As reported previously, people who consumed approximately 0.7 mg/day of BCX had a significantly lower risk of lung cancer (9). Final body weight remained unchanged and did not show a statistically significant difference among all groups in both BCO1−/−BCO2−/− and WT mice. This observation is important because it suggests that the pathological and molecular changes we investigated are due solely to damaging effects of CS-exposure (38). It has been established that an oxidative burden, such as cigarette smoke, can cause weight loss in murine models (39). It is possible we did not see weight changes due to the short-term nature CS-exposure. A previous report did show sex differences in carotenoid absorption following fruit and vegetable consumption (40), however we did not observe any sex difference in terms of BCX accumulation and vitamin A levels in both BCO1−/−BCO2−/− and WT mice. This could be due to the short-term nature of the study and the controlled and equal administration of BCX supplementation to the mouse diet to prevent any
confounder that may have been present in human epidemiological studies, such as the finding that women are more likely to consume fruits and vegetables rich in carotenoids over men (41).

BCX feeding resulted in a significant hepatic accumulation of BCX (30-43 nmol BCX/g liver) in $BCO1^{-/-}/BCO2^{-/-}$ DKO mice, but was non-detectable in non-supplemented mice, measured by HPLC. We monitored BCX levels in the liver because total carotenoid concentrations have been shown to be highest in the liver, compared to other tissues in humans (42), as well as reserving the limited amount of mouse lung tissue samples for other experimental outcomes in this study. Because both WT and DKO mice received equivalent amounts of BCX (20 mg/kg diet) in their diets, we were able to confirm our mouse model of studying intact BCX by measuring hepatic BCX, retinol, retinyl ester and retinoic acid levels in the liver of WT mice compared to DKO. We found that similar concentrations of retinol in the liver were detected in mice with or without BCX supplementation, because of the lack of BCO1 and BCO2 enzymatic cleavage in DKO mice, and only in WT mice, there was a significant change in the generation of retinol. Therefore, confirming our DKO model, is working, in that BCX is unable to be cleaved to form vitamin A in the liver. Although data for quantifying BCX concentration in human liver samples is lacking, one study with a small number of human cadavers reported lower hepatic cryptoxanthin levels ($\sim 2.3 \pm 4.8$ nmol cryptoxanthin/g liver) (42). This large difference compared to the DKO mice can be attributed to the fact that: 1) We are using $BCO1^{-/-}/BCO2^{-/-}$ DKO mice, therefore we expect to see a significant increase in accumulation of BCX, without a change in vitamin A status. Moreover, Amengual et al showed BCX metabolism requires BCO2, and BCO2 ablated mice
accumulated BCX in the liver (24); 2) We are feeding our mice a diet supplemented with BCX versus measuring carotenoids in human liver without any type of dietary information or known consumption of any or all carotenoids prior to subject death; 3) Although within physiologically-attainable range, we administered pure dietary BCX supplementation in order to monitor the molecular changes due to intact BCX, therefore increased accumulation is expected; and; 4) Previous reports show discrepancies between murine models and human absorption of carotenoids (28). Moreover, when comparing this human data to our WT mice, we see a much smaller scope of differences in hepatic BCX accumulated with or without CS-exposure. Thus, further confirming the efficiency of our DKO model to study intact BCX, without generation of vitamin A or β-apo-10’-carotenoids.

We did observe a significant decrease in the level of hepatic retinol in CS-exposed groups, regardless of BCX supplementation in BCO1−/−BCO2−/− mice, but not in WT mice. Previous studies have shown retinol levels in human plasma decreased with CS-exposure (43). The large oxidative burden of CS-exposure has been shown to decrease the stability of retinol in plasma (43), thereby affecting its transport to and concentration in tissue. Although this impact that CS-exposure has on the stability of vitamin A in BCO1−/−BCO2−/− mice warrants further investigation, a major source of preformed vitamin A and storage for of retinol in the liver, retinyl palmitate (46), remained unchanged among all groups, in both DKO and WT, further confirming our ability to study the biological activities of intact BCX, without the generation of vitamin A.
BCX supplementation was able to significantly decrease CS-induced infiltration of inflammatory cells in the lungs of mice, compared to mice given a control diet in both $BCO1^{-/-}/BCO2^{-/-}$ and WT mice. We then investigated intact BCX in $BCO1^{-/-}/BCO2^{-/-}$ mice at the molecular level. BCX supplementation significantly decreased CS-exposure upregulation of IL6 and TNF-α mRNA expression, compared to mice on control diets without BCX. These results show that intact BCX, not metabolites of BCX from BCO1 and BCO2 cleavage, possesses anti-inflammatory activities, in response to CS-exposure. These findings are in concert with a previous study in our laboratory that demonstrated BCX ability to decrease tobacco-specific carcinogen, 4-nitrosamino-1-(3-pyridyl)-1-butane (NNK)-promoted and nicotine-driven IL-6 mRNA expression in the lung (6).

A trademark of CS-induced emphysema is the structural changes to the lung (47,48). Two common indicators of this structural damage include the percentage of hyperplastic bronchiolars and the average distance between alveolar airspace membranes (Lm) in the lung (35). Our model showed a significant increase of both hyperplastic bronchiolars and Lm in response to CS-exposure, compared to control mice in both $BCO1^{-/-}/BCO2^{-/-}$ and WT mice. We did examine the lungs of the DKOs for the presence of tumors when we sacrificed them and did not find any with or without CS, despite the increase in percentage of bronchiolars with hyperplastic epithelium in the pathology report. We did not detect lymphoid follicles by hematoxylin staining; therefore, we did not perform IHC to detect CXCR3-expressing T or B cells as indication of tumor formation or tumor foci or lymphoid follicles in the lungs.

We then investigated intact BCX in $BCO1^{-/-}/BCO2^{-/-}$ mice at the molecular level. Molecularly, these pathological changes in $BCO1^{-/-}/BCO2^{-/-}$ mice were in correspondence
with a significant increase in matrix metalloproteinases, MMP-2 and MMP-9, mRNA expression in the lung. BCX supplementation significantly decreased both the damaging structural changes in CS-exposure, as well as the corresponding molecular changes. MMP-2 and MMP-9 are proteases which, when produced, function to cleave extracellular matrix and structural proteins in tissue, leading, not only to structural changes, but also having inflammatory implications (49). MMP-2 and MMP-9 secretion is increased in response to CS-exposure (50). Our pathology data indicates that dietary BCX can reduce CS-exposure damage, and that molecularly, BCX decreases this structural damage via attenuating the expression of both MMP-2 and MMP-9. These findings are further supported by a previous study in our laboratory that found BCX was able to decrease MMP-2 following stimulation with PNU-282987, an agonist of alpha7 nicotinic acetylcholine receptor (α7-nAChR) in vitro (14). Furthermore, α7-nAChR upregulation in response to smoking is well-established, and our laboratory also found a decrease in α7-nAChR in NNK-treated A/J mice following BCX supplementation (14). These findings suggest that intact BCX, without the generation of its biologically active metabolites by BCO1 and BCO2, is responsible for conferring protective properties against matrix destruction and inflammation, which are the two major contributing factors in the initiation and progression of lung lesions associated with emphysema and bronchitis.

This is the first time that a study has been able to show that BCX, not metabolites generated from BCX, is able to protect against CS-induced inflammation and structural damage by proteases within the lung. Potential mechanisms by which BCX is functioning to protect the lungs against CS-exposure damage could be that BCX is functioning as a
strong antioxidant against any generation of DNA damage as a result of CS (e.g. double stranded DNA breaks) or that BCX is able to transactivate RAR/RXR directly through retinoid signaling pathway. In addition to demonstrating intact BCX biological activities, this study presents a murine model capable of developing an inflammatory response and pathological changes in the lung in response to short-term CS-exposure. Thus, this model has set the stage for, and should be taken into consideration for future short-term CS-exposure studies. By including WT mice, we were also able to examine the biological activity of BCX in the presence and absence of cleavage enzymes. This is important because of the prevalence of SNPs in humans cause a differential absorption, impacting physiological function, of carotenoids at the population level. Examining WT mice also helps to identify any response that may occur due to CS-exposure or BCX feeding in the mice, due to the genetic modification of knocking out BCO1 and BCO2 in the DKO mice. We found that BCX feeding to mice in CS-exposed groups had the same effect in both the DKO mice and the WT mice. Thus, there was no significant strain effect in the mice. Further, because we identified BCX anti-inflammatory and anti-inflammatory lung lesion development following CS-exposure in DKO and WT mice, we can say with confidence that BCX is able to confer biologically relevant activity in the presence or the absence of cleavage enzymes. Also, because of the lower levels of BCX in the livers of WT mice, but the same effect was found, the data suggests that BCX can confer anti-inflammatory action with lower storage levels in the liver, compared to the BCO1/2 DKO mice.

We then conducted in vitro studies because, previously, we could not detect any apo-carotenoids generated by BCO2 cleavage. Previous studies demonstrated that BCX
in a dose- and time-dependently inhibited cell growth in both premalignant and malignant lung cell lines. Moreover, another study from our laboratory revealed that nicotine driven, NNK-induced lung inflammation and tumorigenesis coincided with decreased protein levels of NAD\(^+\)-dependent deacetylase, Sirtuin 1 (SIRT1), that has been implicated in various biologic processes such as metabolism, inflammation, immune function, and apoptosis (6). In the present study, BCX, at comparable doses to the current study, was able to restore SIRT1 levels, while significantly decreasing lung tumorigenesis and inflammation. Additionally, concentrations ranging from 0.5 to 4 \(\mu\)M inhibited the LPS-stimulated expression of IL-6, TNF-\(\alpha\) and MCP-1 at mRNA in BEAS-2B cells at dose-dependent manner. Moreover, BCX impact on IL-6 expression was associated with increased the expression of the SIRT1 at protein levels and reduced AKT phosphorylation. BCX, not 3OH-BA10C, up-regulated I\(\kappa\)B-\(\alpha\) protein, without affecting Erk1/2 MAPKs, further supporting the anti-inflammatory biological activity of BCX. Because comparable doses of BCX and 3OH-BA10C can attenuate LPS-induced inflammatory responses, the modulation of SIRT1 with BCX against inflammation could be due to biological action of BCX as its own intact molecule. It is unlikely that the inhibition of inflammation by BCX was due to its conversion into vitamin A or apo-10-carotenoids due to the low concentrations of BCX (1 to 4 \(\mu\)M) used in this \textit{in vitro} study. These findings indicate BCX’s ability to attenuate LPS-driven inflammation and support the notion that BCX may have biologically relevant activities, as compared to 3OH-BA10C.

Taken together, we demonstrated that BCX in the presence or the absence of BCO1 and BCO2 cleavage enzymes possesses its own biological activity and prevents
CS-induced inflammation. This study confirms the effectiveness of our model to study both intact and cleaved BCX following dietary preventative intervention. Our study provides strong experimental evidence and described potential mechanisms to explain the significant protective association between a high intake of BCX and the risk of lung diseases in smokers. The findings from this study suggest the powerful anti-inflammatory capabilities of BCX feeding and present data that can be used as preclinical evidence for the development of future clinical studies examining BCX.
**Author Contributions**

R.C., K.H., X-D.W. designed the experiments. R.C. performed the in vivo study and experiments. K.H. performed the in vitro experiments. J.V. provided the mice. C.L. performed the histopathological analysis. R.C., K.H., C.L., X-D.W. performed statistical analysis. R.C. and K. H. prepared figures and table. R.C., J.V., X-D.W. prepared manuscript.
Acknowledgments

We thank Dr. Hansgeorg Ernst of the Fine Chemicals and Biocatalysis Research, BASF, Ludwigshafen, Germany, for providing BCX and 3OH-BA10C. We thank Dr. Irfan Rahman, Dr. Dayong Wu and Dr. Virender Kaushik for their useful comments in the progression of this study.

This study was supported by the NIH/NCI CA176256 grant, and US Department of Agriculture grant 1950-51000-074S and NIFA/AFRI grant 67017-26363. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of National Institute of Health and the U.S. Department of Agriculture.
Literature Cited


37. Welcome to the USDA Food Composition Database [Internet]. [cited 2017 Jul 6]. Available from: https://ndb-nal-usda-gov.ezproxy.library.tufts.edu/ndb/


Tables and Figures
### Table 1.1: Wild Type (WT) and $BCO1^{-/-}/BCO2^{-/-}$ Double Knockout (DKO) Mouse Study Outcomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 mg/kg BCX</th>
<th>20 mg/kg BCX</th>
<th>CS</th>
<th>CS + 20 mg/kg BCX</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Animal (n)</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>WT Average Age (d)</td>
<td>206</td>
<td>206</td>
<td>206</td>
<td>206</td>
</tr>
<tr>
<td>WT Final Body Weight (g)</td>
<td>36.1±9.1 a</td>
<td>36.5±7.2 a</td>
<td>37.9±7.6 a</td>
<td>35.2±6.5 a</td>
</tr>
<tr>
<td>WT Urinary Cotinine (µg/ml)</td>
<td>0.49±0.23 a</td>
<td>0.38±0.21 a</td>
<td>3.89±1.3 b</td>
<td>3.96±3.1 b</td>
</tr>
<tr>
<td>DKO Animal (n)</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>DKO Average Age (d)</td>
<td>200</td>
<td>201</td>
<td>205</td>
<td>210</td>
</tr>
<tr>
<td>DKO Final Body Weight (g)</td>
<td>32.9±8.5 a</td>
<td>34.0±6.3 a</td>
<td>30.7±5.7 a</td>
<td>31.8±7.8 a</td>
</tr>
<tr>
<td>DKO Urinary Cotinine (µg/ml)</td>
<td>0.39±0.35 a</td>
<td>0.23±0.26 a</td>
<td>4.14±2.3 b</td>
<td>4.02±3.2 b</td>
</tr>
</tbody>
</table>

**WT and DKO study outcomes:** Summary of animal information and outcomes including animal number, animal average age, animal final body weight and animal urinary cotinine level. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: $p<0.05$)
**Hepatic BCX, retinol and retinyl palmitate as detected by HPLC:** Hepatic levels of a) BCX, b) retinol, and c) retinyl palmitate in mice, detected by HPLC. Significant accumulation of BCX in mice in BCX-feeding groups. Retinol only significantly increased after BCX feeding in WT mice. Retinyl palmitate only slightly increased in WT, no CS, fed BCX, but overall did not change, regardless of CS or BCX feeding. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05)
Figure 1.2: Cigarette Smoke (CS)-induced inflammation and changes to pulmonary structure in lungs of $BCO1^{-/-}/BCO2^{-/-}$ Double KO mice (DKO)

**CS-induced inflammation and changes to pulmonary structure in lung of DKO mice:** Representative images of H&E stained lung (Four μm sections). A, normal alveoli and bronchiolar. B, alveolar airspace enlargement, C. bronchiolar with hyperplastic epithelium, D. alveolar infiltration of inflammatory cells. H&E, hematoxylin and eosin
**Figure 1.3:** β-Cryptoxanthin (BCX) feeding significantly prevented inflammation in the lung of wild type (WT) mice and $BCO1^{-/-}/BCO2^{-/-}$ double knock out (DKO) mice and exposed to cigarette smoke (CS)

*BCX feeding significantly prevented inflammation in the lung of WT and DKO mice exposed to CS:* Quantification of H&E stained lung pathology revealed a significant decrease in, A. neutrophil accumulation ($p<0.0001$), and B. macrophage accumulation ($p<0.0001$). Lung inflammation, macrophages and neutrophils were quantified in the alveoli for 10 fields at a magnification of 400X (1.59 mm$^2$) for each animal, and the number of macrophages and neutrophils was expressed as cells/mm$^2$. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: $p<0.05$)
Figure 1.4: β-Cryptoxanthin (BCX) feeding significantly prevented inflammation, at the molecular level, in the lung of $BCO1^{-/-}/BCO2^{-/-}$ double knockout (DKO) mice exposed to cigarette smoke (CS).

**BCX feeding significantly prevented inflammation, at the molecular level, in the lung of DKO mice exposed to CS:** Beta actin was used as control for the following: B. mRNA expression of TNF-α (p<0.001), C. mRNA expression of IL-6 (p<0.01). Values are expressed as mean ± SEM and CS group (n=9), BCX+CS group (n=8). Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05).
Figure 1.5: β-Cryptoxanthin (BCX) supplementation significantly inhibited cigarette smoke (CS)-exposed induced lung structural pathology changes in wild type (WT) mice and $BCO1^{-/-}/BCO2^{-/-}$ double knockout (DKO) mice.

**BCX supplementation significantly inhibited CS-exposed induced lung structural pathology changes in WT and DKO mice.** Quantification of H&E stained lung pathology revealed a significant decrease in, A. percentage of hyperplastic bronchiolars ($p<0.0001$). Hyperplastic bronchioles of the epithelium were examined for 20 fields at a magnification of 100X (0.63 cm$^2$) for each animal and expressed as a percentage (%) (the number of bronchiolars with hyperplastic epithelium divided by the total number of bronchiolars examined). B. average distance between alveolated surfaces, Lm ($p<0.0001$). Measurement of Lm was performed by using a 100×100 μm grid that was randomly positioned over each field in the lung. The total length of each line of the grid divided by the number of alveolar intercepts yielded the average distance between alveolated surfaces, or the Lm. For each animal, 10 fields at a magnification of 200X were examined. Beta actin was used as control for the following. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: $p<0.05$)
Figure 1.6: β-cryptoxanthin (BCX) supplementation significantly inhibited cigarette smoke (CS)-exposed induced matrix metalloproteinase (MMP) expression, at the molecular level, in the lungs of $BCO1^{-/-}/BCO2^{-/-}$ double knockout (DKO) mice.

BCX supplementation significantly inhibited CS-exposed induced MMP expression, at the molecular level, in the lungs of DKO mice: mRNA expression of MMP-2 ($p<0.03$), C. mRNA expression of MMP-9 ($p<0.02$). Values are expressed as mean ± SEM and CS group (n=9), BCX+CS group (n=8). Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: $p<0.05$).
Figure 1.7: β-Cryptoxanthin (BCX) treatment reduced inflammation, increased Sirtuin 1 (SIRT1) protein and decreased phosphorylation of Protein kinase B (pAkt/Akt) in lipopolysaccharide (LPS)-stimulated BEAS-2B cells

**BCX treatment reduced inflammation, increased SIRT1 protein and decreased pAKT in LPS-stimulated BEAS-2B cells:** A. mRNA expression of IL-6, TNF-α, MCP-1 decrease, B. SIRT1 protein levels increase, C. p-Akt protein levels decrease, D. IκBα protein levels increase, all in a dose-dependent manner with BCX pre-treatment (0.5 to 4 μM) following LPS stimulation (10 ng/ml). Beta actin and total-Akt were used as controls. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05). BEAS-2B, human bronchial epithelial cells; TNF, tumor necrosis factor; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; SIRT1, sirtuin1; AKT, Protein kinase B; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; LPS, Lipopolysaccharide
Figure 1.8: β-Cryptoxanthin (BCX) metabolite, 3-OH-β-apo-10'-carotenal (3OH-BA10C) protected against inflammation, increased Sirtuin 1 (SIRT1) protein and decreased phosphorylation of Protein kinase B (pAkt/Akt) in lipopolysaccharide (LPS)-stimulated BEAS-2B cells

A

![Bar graph showing mRNA expression of IL-6, TNF-α, MCP-1 decrease, B. SIRT1 protein levels increase, C. p-Akt protein levels decrease, D. IκBα protein levels increase, all in a dose-dependent manner with 3OH-BA10C pre-treatment (0.5 to 4 μM) following LPS stimulation (10 ng/ml). Beta actin and total-Akt were used as controls. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05). BEAS-2B, human bronchial epithelial cells; TNF, tumor necrosis factor; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; SIRT1, sirtuin1; AKT, Protein kinase B; LPS, Lipopolysaccharide.]

BCX metabolite 3OH-BA10C protected against inflammation, increased SIRT1 protein and decreased pAkt in LPS-stimulated BEAS-2B cells: A. mRNA expression of IL-6, TNF-α, MCP-1 decrease, B. SIRT1 protein levels increase, C. p-Akt protein levels decrease, D. IκBα protein levels increase, all in a dose-dependent manner with 3OH-BA10C pre-treatment (0.5 to 4 μM) following LPS stimulation (10 ng/ml). Beta actin and total-Akt were used as controls. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05). BEAS-2B, human bronchial epithelial cells; TNF, tumor necrosis factor; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; SIRT1, sirtuin1; AKT, Protein kinase B; LPS, Lipopolysaccharide.
Chapter 2
Mechanistic Role of Sirtuin 1 Enzymatic Activity in Cigarette Smoke-Induced Emphysema

Rachel A. Chiaverelli¹², Chun Liu¹, Dayong Wu³, Irfan Rahman⁴, Virendar Kaushik⁵, Isaac K. Sundar⁴, Michael McBurney⁶, Xiang-Dong Wang¹²*

1. Nutrition and Cancer Biology Lab, Jean Mayer USDA-HNRCA at Tufts University, Boston, MA, USA
2. Gerald and Dorothy J. Friedman School of Nutrition and Science Policy, Tufts University, Boston, MA, USA
3. Nutritional Immunology Lab, Jean Mayer USDA-HNRCA at Tufts University, Boston, MA, USA
4. Department of Environmental Medicine, Lung Biology and Disease Program, University of Rochester Medical Center, Rochester, NY, USA
5. Center for the Development of Therapeutics, Broad Institute of MIT and Harvard, Cambridge, MA, USA
6. Department of Medicine, Microbiology and Immunology Lab, University of Ottawa, Ontario, Canada

*Corresponding Authors:
Xiang-Dong Wang, MD, PhD
Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University
711 Washington Street, Boston, MA 02111
Phone: 617-556-3130 or 3144   Fax: 617-556-3344 or 3224
Email: xiang-dong.wang@tufts.edu
Abstract

Cigarette smoking (CS) remains a major public health problem and is a leading cause of emphysema onset, and the progression to permanent damage observed in chronic obstructive pulmonary disease (COPD). Currently, the underlying mechanism by which CS-exposure induces inflammation and inflammatory lung lesions remains unclear. Moreover, SIRT1 has been shown to play a controversial role in both CS-induced inflammation and inflammatory lung lesion development. In the present study, we aim to investigate the mechanistic role that SIRT1 enzymatic activity plays in cigarette smoke (CS)-induced inflammation and emphysema. We hypothesized that administering six weeks of CS to male and female whole body Sirt1<sup>y/y</sup> homozygous mutant mice, without SIRT1 enzymatic activity, Sirt1<sup>+/y</sup> heterozygous mutants, with partial SIRT1 activity, and wild type (WT) littermates (Sirt1<sup>+/+</sup>), would induce inflammation and emphysema, and that Sirt1<sup>y/y</sup> mice would exhibit an exacerbation of pulmonary lesions due to an increase in inflammatory response to CS at both the pathological and molecular levels. We measured the pathological grade of inflammation based on characteristics of inflammation and cases of emphysema in the lung by hematoxylin and eosin (H&E) staining. Inflammation significantly increased in Sirt1<sup>+/+</sup> and Sirt1<sup>+/y</sup> given CS, as expected, but unexpectedly, Sirt1<sup>y/y</sup> mice did not show any increase in inflammatory grade in the pulmonary pathology report given CS-exposure. Molecularly, no significant change with CS-exposure was observed in expression of TNF-α or IL-10, however IL-6 expression decreased with CS, indicating a possible anti-inflammatory role. Despite a lack of inflammation, Sirt1<sup>y/y</sup> mice developed more cases of emphysema compare to that of Sirt1<sup>+/+</sup> and Sirt1<sup>+/y</sup> mice. Surprisingly, gene expression of matrix metalloproteinase
expression (MMP-12, MMP-2, MMP-9) decreased due to CS-exposure across genotypes to the same extent. Interestingly, across all genotypes exposed to CS, we observed a decreased gene expression of nicotinamide phosphoribosyltransferase (NAMPT), a key enzyme in the synthesis and proper recycling of NAD⁺, possessing many implications in inflammation and the aging lung. We then discovered that p53, a transcription factor associated with cell cycle progression was significantly decreased with CS. Furthermore, cyclin-dependent kinase 1 (CDK1), a major player in cell cycle progression was significantly decreased and cellular senescence (p16, p21) was significantly increased in CS-exposed Sirt1⁺/⁺ and Sirt1⁻/⁻ mice. This suggests cell arrest and cellular senescence may be playing in emphysema development, dependent on SIRT1 enzymatic activity. To conclude, mice with a homozygous mutation of SIRT1 enzymatic activity do not produce the same inflammatory response to CS compared to WT mice, yet have an increase in emphysema cases, independent of MMP expression. Molecular data provides insights that NAMPT may be a sensitive target to CS-exposure, impacting the observed impaired inflammatory response and decreased transcription factors related to cell cycle progression and subsequent increases in cellular senescence with a loss of SIRT1 enzymatic activity. This study presents insight for future in vivo work investigating the complex interrelationship between NAMPT, NAD recycling and SIRT1 enzymatic activity and their roles in cellular senescence due to CS-exposure, leading to emphysema development. Elucidating the interplay between these enzymatic players could provide an avenue for an effective metabolic target in the prevention of emphysema and COPD.
**Introduction**

Despite numerous campaigns to reduce the prevalence of cigarette smoking, it remains a major public health problem (1). Rates of smoking related deaths continue to rise, cause about one in five deaths each year in the United States (2). Cigarette smoke (CS) presents a strong oxidative burden composed of a complex mixture of harmful toxins that do not discriminate against pathophysiological and molecular targets (3,4). Consequently, the oxidative burden of CS is able to induce a strong inflammatory response (5,6), encompassing a vast network of cytokines which are linked to a wide range of diseases, specifically lung diseases (7).

Inflammation due to CS is known to induce the generation of inflammatory lung lesions and emphysema (5,6,8). In brief, the current understanding of CS-induced inflammation and emphysema is that the oxidative burden of CS causes the secretion of chemokines from the epithelial cell lining of the alveolar membrane in the lung, and this release stimulates the recruitment of inflammatory cells, predominantly neutrophils and macrophages to the lung. The infiltration of these inflammatory cells, work with the adaptive immune system to respond to the oxidative burden of CS, and in turn, a cascade of inflammatory responses occurs stimulating further recruitment of inflammatory cells, and provoking the release of protease, which to cleave matrix proteins within the lung, thereby creating alveolar airspace enlargement and destruction, which eventually develops into emphysema (Figure 1) (9,10). Proteases that contribute to the destruction of the pulmonary alveolar membrane are primarily matrix metalloproteinases (MMPs). Differential secretion and expression of MMPs has classically been associated with development of lung lesions and emphysema (11–14). Chronically, this may developed
into irreversible pulmonary damage and chronic obstructive pulmonary disease (COPD) (15,16).

In spite of current knowledge of inflammation-driven lung lesions in the development of emphysema, much is still unknown about the underlying mechanism by which this occurs. Sirtuin 1 (SIRT1), a class Ia mammalian sirtuin localized in the nucleus, cytoplasm and mitochondria linked to anti-aging properties (17,18). SIRT1 functions as a histone deacetylase (HDAC) and its enzymatic activity is dependent on nicotinamide adenine dinucleotide (NAD)+ recycling, therefore, SIRT1 is a very sensitive target to changes in redox due to endogenous sources such as CS (19–22). Moreover, decreased plasma SIRT1 levels have been identified in COPD patients (23).

It is important to note, however, that SIRT1 protein and/or enzymatic activity level do come with some controversial findings from the laboratory. Because of its ability to deacetylate many substrates, SIRT1 has had implications in the protection from various diseases, as well as the promotion of disease (17,24,25). Thus, SIRT1 and its close relationship to CS-induced inflammation, and its clinical relevance to patients suffering from COPD, must be better understood in a preclinical model which specifically examines the whole-body enzymatic activity of SIRT1 in relation to CS-exposure.

In the present study, we aim to elucidate the complex underlying mechanism by which SIRT enzymatic activity contributes to CS-induced inflammation and emphysema development in mice. We utilize a novel, genetically modified mouse model in which SIRT1 enzymatic activity had been completely ablated (homozygous mutation, Sirt1<sup>y/y</sup>), partially ablated (heterozygous mutation, Sirt1<sup>+/-</sup>), and fully functioning SIRT1 (Wild
type, WT, Sirt1^{+/+}) to demonstrate the deleterious, inflammation-inducing effects of CS-exposure. This will allow us to contribute to the current preclinical scientific understanding of SIRT1 role in these processes, and have implications in future work that focus on SIRT1 as a mechanistic target in emphysema and COPD.
Materials and Methods

1. Animal Study and Design

All study protocols including breeding were approved by the Institutional Animal Care and Use Committee of Tufts University. We used SIRT1 homozygous \((Sirt1^{+/+})\) and SIRT1 heterozygous \((Sirt1^{+/-})\) mutant mice with a 129/SvJ background expressing the SIRT1 protein with no catalytic activity due to a mutation on H355Y, as well as their wild type \((Sirt1^{+/+})\), 129/SvJ background counterpart mouse. At 4 weeks of age, SIRT1 genotypes of animals were determined by a PCR-based test performed on DNA isolated from tail-tip biopsies using the primers 5’-TGGAAGGAAAGCAATTTTGGT-3’ and g’CTGAGTTACCTTAGCTTGGC-3’. Mice were fed the standard laboratory chow diet (Envigo, Huntingdon, UK). At seven months (28 weeks) of age, mice were randomly assigned into control or CS-exposed groups. Mice in CS-exposed groups received CS for six weeks, as previously described (26). Briefly, mice receiving CS-exposure were placed in individual plastic containers with sufficient ventilation to allow CS to enter. Contained mice were exposed to CS within our laboratory’s CS-chamber, which consists of an anaerobic chamber created by members of our laboratory who fused to tubing that connects to an apparatus capable of holding 10 research cigarettes at a time. The CS-exposure schedule was as follows: Week one: 20 cigarettes per day (10 in the morning, 10 in the afternoon); Weeks two-six: 40 cigarettes per day (20 in the morning, 20 in the afternoon). The amount of CS-exposure was determined by urinary cotinine equivalents, a stable metabolite of nicotine (27), and was similar to that found in humans smoking approximately 1.5 packages of cigarettes per day. Cotinine levels in the urine were measured by ELISA (ABNOVA, Taipei, Taiwan). All mice were
killed at eight and a half months (34 weeks) of age by cardiac puncture under deep isoflurane anesthesia.

2. Pathology

The right upper lobe of each lung was inflated and fixed by gentle intratracheal infusion of 10% neutral buffered formalin at an average rate of 15 cm of H₂O pressure for 2 minutes, and then immersed in fresh 10% neutral buffered formalin for a period of 72 hours. The samples were subsequently processed and embedded in paraffin, and serially sectioned. Four μm sections were stained with hematoxylin (H) and eosin (E) for histopathological examinations.

The degree of severity of lung inflammation was estimated by peribronchial, perbronchiolar and perivascular infiltrates of inflammatory cells, as well as alveolar septal infiltrates as follows: grade 0 (none), no inflammation; grade 1 (+) (normal/minimal), occasional cuffing with sporadic inflammatory cells for bronchi, bronchioles and blood vessels; grade 2 (++) (mild), a thin layer of peribronchial, peribronchiolar, and perivascular cuffing with inflammatory cells and a few inflammatory cells of alveolar septal infiltrates; grade 3 (+++) (moderate), a moderately thick layer of peribronchial, peribronchiolar, and perivascular cuffing with inflammatory cells and some inflammatory cells of alveolar septal infiltrates; and grade 4 (++++) (severe), a thick layer of peribronchial, peribronchiolar, and perivascular cuffing with inflammatory cells and several inflammatory cells of alveolar septal infiltrates. For each animal, 20 fields at a magnification of 100X were examined.
Pulmonary emphysema grading was based on a grading system from 0 to 3 in which grade 0 represented no emphysema development, grade 1 showed focal area emphysema, grade 2 indicated multifocal emphysema, and grade 3 indicated diffuse emphysema. For each animal, 20 fields at a magnification of 100X were examined.

The sections were blindly examined by independent investigators by light microscopy [ZEISS Microscopy, PixeLINK USB 2.0 (PL-B623CU) Camera, PixeLINK μScope Microscopy Software].

3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TriPure Isolation Reagent (Roche Applied Science). cDNA was prepared using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and an automated thermal cycler PTC-200 (MJ Research, Bio-Rad Laboratories, Hercules, CA). qRT-PCR was carried out using Fast Start Universal SYBR Green Master (ROX) (Roche, Indianapolis, IN). Relative gene expression was determined using the \(2^{-\Delta\Delta C_t} \) method.

4. Statistical Analysis

Measurements are expressed as the mean ± the SEM, unless otherwise indicated. Comparisons across multiple groups were conducted by one-way ANOVA with Tukey Honestly Significant Difference (HSD). Differences between two groups were analyzed by Student t test. Analyses were performed using GraphPad Prism (Version 7.01).
Results

1. SIRT1 enzymatic mutation lacks inflammatory response to CS-exposure

   Final body weight remained unchanged in all groups of mice, regardless of CS-exposure or genotype. Cotinine significantly increased in mice that were exposed to CS. (Table 1). Overall, the inflammation grade significantly (p<0.05) increased in Sirt1+/+ and Sirt1+/y given CS, as expected, but unexpectedly, Sirt1y/y mice did not show any increase in inflammatory grade in the pulmonary pathology report given CS-exposure. CS-exposure significantly increased the overall grade of inflammation in Sirt1+/+ and Sirt1+/y mice, however there was no increase in inflammation in the Sirt1y/y mice. In fact, no inflammation was detected in lung pathology of the Sirt1y/y mice following CS-exposure. (Table 2.1). Molecularly, no significant change with CS-exposure was observed in expression of TNF-α or IL-10, however IL-6 expression decreased with CS, indicating a possible anti-inflammatory role (Figure 2.1).

2. CS-exposure increased cases of emphysema and this effect was exacerbated in mice without SIRT1 enzymatic activity

   CS-exposure significantly (p<0.05) increased emphysema grading in all mice, regardless of SIRT1 enzymatic activity, compared to Sirt1+/+ mice without CS-exposure. Interestingly, Sirt1y/y developed a significantly (p<0.05) more severe grade of emphysema given CS-exposure, compared to mice with partial SIRT1 enzymatic activity (Sirt1+/y) and Sirt1+/+ mice (Table 2.1). Matrix metalloproteinase (MMP-12, MMP-2, MMP-9) expression, however, decreased due to CS-exposure (Figure 2.2).
3. NAMPT decrease in response to CS-exposure regardless of genotype, effecting cell cycle progression and cellular senescence

CS-exposure significantly (p<0.05) decreased gene expression of nicotinamide NAMPT, a key enzyme in the synthesis and proper recycling of NAD, in the lung of \textit{Sirt1}^{+/y}, \textit{Sirt1}^{+/y}, and \textit{Sirt1}^{+/+} exposed to CS, compared to \textit{Sirt1}^{+/+} without CS (Figure 2.3). NAMPT is the rate limiting enzyme for NAD biosynthesis, and due to its important role in metabolism, it is extremely sensitive to changes in redox, such as CS-exposure. We further speculated that this change in NAMPT levels due to CS-exposure, in mice without any SIRT1 enzymatic activity could then have implications in cellular senescence, leading to emphysema (Figure 2.4). Thus, provoking investigation into cell cycle, cell cycle progression, and cellular senescence markers.

4. SIRT1 mutation increased cellular senescence in mice exposed to CS

This effect of CS-exposure on all genotypes coincided with a significant (p<0.05) decrease in p53, key transcription factor involved in cellular proliferation and cell cycle progression (Figure 2.). Furthermore, decrease in cyclin-dependent kinase 1 (CDK1) was observed along with an increase in cellular senescence measured by expression of p21 and p16, direct inhibitors of CK and CDK1, respectively, with decreases in SIRT1 enzymatic activity (\textit{Sirt1}^{+/y} and \textit{Sirt1}^{y/y}) (Figure 2.4D, 2.4E). This suggests cellular senescence may be playing in emphysema development, dependent on SIRT1 activity.
Discussion

In the present study, we examined the effects of six weeks of CS-exposure on mice with no SIRT enzymatic activity (\textit{Sirt1}^{+/y}), partial SIRT1 activity (\textit{Sirt1}^{+/y}), and wild type mice, with full SIRT1 activity (\textit{Sirt1}^{+/+}). Pathological evaluation revealed that, given CS, \textit{Sirt1}^{+/y} mice did not produce and inflammatory response, compared to \textit{Sirt1}^{+/+} and \textit{Sirt1}^{+/y} mice. This result was unexpected, as we expected to see an increase in inflammation with a loss of SIRT1 enzymatic activity based on previous reports that showed SIRT1 levels are protective against inflammation from CS, or other oxidative burdens (19,22,28,29). Moreover, previous work showed that loss of SIRT1 increased inflammation (30,31). Our results were somewhat surprising initially, however this cycles back to the concept of differentiating SIRT1 protein levels versus enzymatic activity. Enzymatic deficiency has been shown to have an impaired immune and autoimmune response (30). It is possible that this generation of emphysema in the absence of an increase in inflammation in the \textit{Sirt1}^{+/y} mice could be because the mice have attained a “maximum level” of inflammation during the course of the study, and therefore at their sacrifice, we are unable to detect any measurable level of inflammation in the presence of the vast amount of damage. Moreover, in unpublished data from our laboratory, when the same strain and enzymatic deficient mice we permitted to age to 10 months, they all developed emphysema naturally. We are currently investigating the mechanisms by which this naturally, age-related emphysema development has occurred, and we plan to compared the findings to this study in order to better understand the mechanism of CS-exposed damage.

We observed a significant decrease in NAMPT given CS, regardless of genotype. This finding is important because of major role that NAMPT plays as the rate limiting
enzyme for NAD biosynthesis, and recycling, which is required for adequate SIRT1 enzyme deacetylase activity (17,18,32). Previous reports have showed that NAMPT, given its role in metabolism, is very sensitive to oxidative burdens, such as CS (33). Changes in NAMPT have been reported to have many inflammatory physiological consequences, as well as work closely with the regulation of SIRT1 enzymatic function (34). We, therefore, believed that loss of SIRT1 enzymatic activity would further impacted NAMPT expression in the lung, but this was not that case. This finding could provide further rationale for why we did not observe an increase in inflammation in \textit{Sirt1}^{v/y} mice, despite the increase in cases of emphysema.

The profound finding of our study is that a loss of SIRT1 enzymatic activity leads to a significant increase in the gene expression of markers of cellular senescence with CS-exposure, therefore demonstrating that SIRT1 activity may play a major role in cellular senescence. Furthermore, there was an observed decrease in CDK1, providing more evidence for cell cycle arrest. Upstream from this, CS-exposure significantly decreased gene expression of p53, major transcription factors with many roles, including major roles in cell cycle progression (35–37). Previous reports have shown an interplay between CS-exposure, SIRT1 and cellular senescence (38–40), however this is the first study of its kind to demonstrate the loss of SIRT1 enzymatic function impairs an expected inflammatory response to CS, yet still develops emphysema with significant changes to cell arrest and senescence at the molecular level (Figure 2.5).

Our study does not come without limitations. One limitation to our work is the background strain of mice used (8,41,42). Previous preclinical work has shown that mouse strains C57BL6/J, A/J and SJ/L have been established as models with predictive
emphysema development following CS-exposure that closely emulated how humans develop CS-induced inflammation and emphysema (41). We used mice with a 129SvJ background, which, based on previous work, may have a resistance to producing an inflammatory response due to CS-exposure (42). Another possible limitation to our study is the length of time we chose to expose our mice to cigarette smoke. Previous studies have demonstrated emphysema development in murine models that require at least six months of CS-exposure (20,43). Our rationale for exposing mice to six weeks of smoke exposure was two-fold: 1) We were unsure of the ability of the mice to cope with the severe oxidative burden of CS, given their lack of SIRT1 enzymatic activity, and SIRT1’s expansive role in the body, in response to oxidation, and 2) We chose to begin mice at seven months of age based on some preliminary data from our lab that showed Sirt1<sup>+/−</sup> mice developed emphysema at 10 months of age naturally, therefore we aimed to study the development of CS-driven emphysema, not purely age-related emphysema, while still maintaining an appropriate model for emphysema. Additionally, due to lack of mouse lung tissue, we were unable to measure the protein levels of markers of interest in the lungs. Further studies are necessary to validate our mRNA expression findings.

Taken together, SIRT1 enzymatic activity’s role in CS-exposure may have a more direct role in the development of emphysema, due to an increase in cell arrest and senescence, independent of an inflammatory response or increased MMP expression. These results further dictate the importance of examining SIRT1 enzymatic activity and function, as opposed to protein levels of SIRT1 in previous knock out or knock in models. The current study presents controversial results to the present scientific knowledge surround SIRT1 as a target of CS-exposure to produce inflammation and
emphysema, as well as the related molecular understanding of both of these processes. To conclude, the data from this study shows loss of SIRT1 enzymatic activity plays a major role in emphysema development due to CS, independent of inflammation, and the underlying mechanism for this finding is the increase in cell arrest and senescence. Therefore, SIRT1 enzymatic activity serves a protective role against CS-induced emphysema driven by cellular senescence.
Acknowledgments
This study was supported by the NIH/NCI CA176256 grant. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of National Institute of Health and the U.S. Department of Agriculture.
Literature Cited


Tables and Figures
Mouse study outcomes: Animal information and study outcomes including mouse number, M:F ratio, final body weight, and cotinine. No differences were observed in final body weight of mice, regardless of genotype of CS-exposure. Urinary cotinine significantly increased in mice exposed to CS. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05)
Table 2.2: Pathological Report of Emphysema Cases and Inflammation Grade

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>CS-EXPOSURE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sirt1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Sirt1&lt;sup&gt;++/+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Emphysema Cases</td>
<td>0% (0/12)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0% (0/9)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inflammation Grade</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Pathology report of emphysema cases and inflammation grade:** Pathology revealed a significant increase in cases of emphysema in *Sirt1<sup>++/++</sup>* mice, independent of inflammation, given CS, by H&E staining. Significant differences between groups were analyzed by a Fisher’s exact test, compared to *Sirt1<sup>++/++</sup>* (emphysema case) or by one-way ANOVA (inflammation grade) and are denoted by different letters (Significance: p<0.05). CS, Cigarette smoke; H&E, Hematoxylin and eosin.
mRNA expression of inflammatory markers IL-6, TNF-α, IL-10: CS-exposure significantly decreased IL-6 expression, regardless of genotype, however, did not significantly impact other markers related to inflammation. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05). Cs, cigarette smoke
**mRNA expression of MMP-12, MMP-2 and MMP-9:** CS-exposure significantly decreased MMP-2 and MMP-9 expression, regardless of genotype. CS-exposure significantly decreased expression of MMP-12 only in Sirt1^{+/y} and Sirt1^{y/y} mice. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05). CS, Cigarette smoke
**mRNA expression of NAMPT:** CS-exposure significantly decreased NAMPT expression, regardless of genotype. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05). CS, Cigarette smoke
Figure 2.4: Potential mechanisms by which CS-exposure impacts NAMPT, leading to emphysema in \textit{Sirt1} \textit{y/y} mice

Modified from Barnes. \textit{J Allergy Clin Immunol.} 2016
mRNA expression of markers of cell cycle progression and cell senescence: CS-exposure significantly decreased p53 in Sirt1^{+/y} and Sirt1^{y/y} mice. p53 is important transcription factors in cell cycle progression. CS-exposure also significantly decreased mRNA expression of cyclin-dependent kinase (CDK)-1, a protein necessary for cell cycle progression, in Sirt1^{+/y} and Sirt1^{y/y} mice. CS-exposure significantly increased mRNA expression of cellular senescence markers, p21 and p16. Significant differences between groups were analyzed by one-way ANOVA and are denoted by different letters (Significance: p<0.05)
**Conclusion:** Whole-body loss of SIRT1 enzymatic activity results in the exacerbated CS-induced emphysema with much less of an inflammatory response, as compared with WT mice. CS-induced decrease in transcription factors related to the regulation of cell cycle progression (p53). Consistent with these findings, we revealed a decrease in cell cycle progression (cyclin-dependent kinase 1, CDK1) and an increase in biomarkers for cell arrest/cellular senescence (p21 and p16), which is likely the underlying mechanism for emphysema development, independent of inflammation. CS, Cigarette smoke
Summary and Discussion

The primary goals of thesis doctoral thesis project were to explore CS-induced inflammation and inflammatory lung lesion development in two genetically modified mouse models in order to demonstrate the powerful anti-inflammatory effect of BCX, as well as better understand the role the SIRT1 enzymatic activity has in vivo.

First, we demonstrated the anti-inflammatory properties of BCX intervention in male and female BCO1^{-/-}/BCO2^{-/-} mice. This was conducted by first by feeding mice with 20 mg/kg diet BCX for two weeks, and then exposing mice to CS for two more weeks. By utilizing mice which have both cleavage enzymes knocked out, we are able to study the effects of intact BCX, independent of its cleavage metabolites, vitamin A by BCO1 and apo-carotenoids by BCO2.

Then, to better understand the mechanism by which the CS-induced inflammation and inflammatory lung lesion development occurred, we utilized a genetically modified mouse model in which SIRT1 enzymatic activity has been completely ablated (Sirt1^{-/-}) mice. We observed a lack of inflammatory response in SIRT1 mutation mice, compared to WT, given CS, however Sirt1^{-/-} developed more cases of emphysema than WT mice exposed to CS. We then discovered a decrease in expression of transcription factor p53, which plays a significant role in cell cycle progression at the molecular level. We found a decrease in CDK1, a downstream kinase necessary for cell cycle progression, and an increase in markers of cellular senescence (p16 and p21). The data from this study shows loss of SIRT1 enzymatic activity plays a major role in emphysema development due to CS, independent of inflammation, and the underlying mechanism for this finding is the
increase in cell arrest and senescence. Therefore, SIRT1 enzymatic activity serves a protective role against CS-induced emphysema driven by cellular senescence.

The first part of this thesis work lays the ground work for by providing more preclinical evidence for the planning and execution of a future clinical study in which physiologically attainable amounts of BCX are given to humans to help with lung disease amelioration, or other diseases related to possibly to other diseases related to inflammation. Then, the second part of this thesis provides preclinical evidence that the enzymatic deacetylase activity of SIRT1 has implications in inflammatory response to an endogenous oxidative agent, such as CS.

Taken together, the results from each part of this thesis provide some critical findings for the scientific community to review and take into consideration when others are designing their studies. One finding being that background strain of the mice really plays a major role in the outcomes of the study. In part one of this thesis, the C57BLJ mouse pathology had a large increase in the infiltration of inflammatory cells given CS-smoke. These results coincided with gene expression of cytokine markers at the molecular level. On the other hand, part two of this thesis work, in which mice were bred on a 129SvJ background, regardless of genetic modification, the mice with CS-exposure did not develop inflammation like that of the C57BLJ mice. The finding concerning discrepancies in inflammatory response, was also evident in the development of inflammatory lung lesions. C57BLJ mice developed significant changes in Lm given CS for just two weeks of exposure. The 129SvJ mice developed emphysema according to pathology after six weeks of CS-exposure, however it was only in the mice with no SIRT1 enzymatic activity that we observed a significant increase in the number of cases.
of emphysema. Previous reports showed that C57BLJ strain mice are very sensitive to endogenous oxidative antagonists, resulting in the development of emphysema at a much shorter time point than that of mice on other background strains. This thesis work further confirmed that important notion. Thus, this thesis provides others with important basic information about the murine model system, and aids others in the future to better develop preclinical studies that will more efficaciously contribute to the scientific literature, in order to provide supportive evidence to enter clinical trials in the future to better assess what is going on in humans and better design effective dietary and pharmacological agents to combat respiratory diseases.

This thesis work gives the scientific community more evidence of the deleterious effects of CS, especially concerning its relationship to the induction of inflammation and inflammatory lung lesions, which lead to lung diseases such as emphysema and, chronically, COPD. Cigarette smoking kills more than 480,000 people each year, and the deaths due to COPD continues to rise. Therefore, this thesis work is extremely important for review of the preclinical scientific community in order to: 1) continue public health pushback against tobacco companies; 2) provide more evidence of the mechanism by which smoking leads to diseases that kill a large number of humans in the U.S. and worldwide each year; 3) demonstrate the effectiveness of dietary intervention with dietary feeding doses within physiological range, and therefore able to easily, and inexpensively modify an individual’s lifestyle to help with the prevention of disease; 4) this work is important for the nutritional education of clinicians with patients suffering from pulmonary diseases and/or inflammation-related diseases.
**Limitations and Strengths**

This thesis work does not come without limitations. In parts one and two, we measure CS-exposure based on cotinine equivalents in urine. Although this has been shown to be a very reliable method of measuring nicotine metabolism, measuring the total particulate matter (TPM) and carbon monoxide in the CS chamber would have further confirmed the oxidative burden from the CS to the mice. TPM and carbon monoxide monitoring are both commonly used in CS studies, but because of the novelty of our CS-chamber, we were unable to accurately measure and confidently report these two pieces of data. We, therefore, relied solely on cotinine in the urine.

Another possible limitation to both parts of our study is the length of time we chose to expose our mice to CS. Previous studies have demonstrated emphysema development in murine models that require at least six months of CS-exposure. Our rationale for exposing mice to two or six weeks of smoke exposure was two-fold: 1) We were unsure of the ability of the mice to cope with the severe oxidative burden of CS, given their lack of BCO1/BCO2 and SIRT1 enzymatic activity, respectively, and 2) We chose to begin smoking mice as six-seven months of age in order to established a model related to gaining, and based on some preliminary data from our lab that showed Sirt1<sup>y/y</sup> mice developed emphysema at 10 months of age naturally, therefore we aimed to study the development of CS-driven emphysema, not purely age-related emphysema, while still maintaining an appropriate model for emphysema.

Data that would have strengthened the conclusions of both parts of this thesis work would have been 1) measuring the protein levels of molecular markers of interest, and 2) conducting a bronchoalveolar lavage (BAL) to collect bronchoalveolar lavage fluid
(BALF) samples from the lungs of our mice. Due to the lack of lung tissues in our mice, from use in pathology, we relied heavily on the monitoring of mRNA expression to indicate changes due to either CS-exposure, BCX feeding, SIRT1 enzymatic activity, or a combination of these factors. Although mRNA expression provides valuable information regarding the molecular changes in lung tissue samples, protein levels, in parallel, would have been valuable information to further elucidate the mechanisms involved in inflammation, inflammatory lung lesions, and, especially, emphysema development in Aim 2. Further work measuring the protein levels is necessary to validate any changes that we observed at the molecular level in the lungs of mice. In addition to collection of protein data, BALF sample collection would allow us to better understand the inflammatory cells that occupy the lung in control mice, and detect any increase in inflammatory cells in the lungs of mice given CS. Due to low mouse numbers, and the fact that following BALF collection it is not advised to perform pathology of the lung structures from possible damage during the collection procedure, we chose not to collect BALF in order to be able to achieve statistical significance between our mouse groups.

In part two, another limitation to our work is the background strain of mice used. Previous preclinical work has shown that mouse strains C57BL6/J, A/J and SJ/L have been established as models with predictive emphysema development following CS-exposure that closely emulated how humans develop CS-induced inflammation and emphysema. We used mice with a 129SvJ background, which, based on previous work, may have a resistance to producing an inflammatory response due to CS-exposure.

Another limitation to part two of this thesis work is the processing of lung tissue during harvesting. We discovered in part two, that cellular senescence is playing a role in
emphysema development, independent of pulmonary inflammation, therefore, in order to better investigate cellular senescence, it would have been beneficial to examine beta-galactosidase activity in the lungs of these mice exposed to CS versus non-CS-exposed mice. In order to accurately measure this activity, the best way is to cryo-preserve the fixed tissue immediately when harvesting the tissues and sacrificing the mice. By doing so, you prevent the loss of any enzyme activity in the lung tissue and you can therefore measure beta-galactosidase as a reliable marker of cellular senescence.

**Future Directions**

Based on this evidence this thesis work presents, future preclinical directions are to further investigate the efficacy of intact BCX in a long-term CS-exposure study. Increasing the time of exposure will allow our group to better investigate the development of emphysema and progression to COPD in our mouse model, and therefore better address BCX ability to prevent CS-induced inflammation, inflammatory lung lesions, emphysema, and COPD. This will allow us to be able to compare our results to what the epidemiological studies are telling us about BCX association with improved outcomes in lung cancer and in COPD patients. By extending the time of CS-exposure, we also are able to investigate the possible induction of lung tumorigenesis, as a result of CS-exposure, and the prevention of tumor onset and progression by BCX. We also can address the underlying mechanisms further by examining lung tissue.

We also plan to extend the CS-exposure in part two of this thesis work, while also adding additional intervention groups that will receive BCX and resveratrol, a well-known activator of SIRT1 enzymatic activity. By making these progressive changes to future work, we add value in a few areas of our research: 1) Extending the time of CS-
exposure provides a better model for studying the underlying mechanisms of emphysema and COPD development as a result of CS; 2) Adding intervention groups allows us to study, not only the underlying mechanisms of emphysema and COPD, but prevention of those pathophysiological outcomes, and the mechanism by which this occurs and if it is SIRT1-dependent; 3) Make relevant comparisons between BCX biological activity in the prevent of CS-induced inflammation, inflammatory lung lesions, emphysema, and COPD to that of resveratrol, which much work has been reported in its biological activity functions as an antioxidant and as an inducer of SIRT1 enzymatic activity. By conducting this future study, we will be able to elucidate the biologically active functions of BCX to better our understanding, and the scientific community’s understanding, as well as provide more preclinical evidence for BCX activity for studies in humans at the clinical level.

In addition to extending CS-exposure and providing an intervention to SIRT1 enzymatically mutated mice, we aim to better understand the inflammatory response in the mouse lung by implementing BAL procedure to collect and evaluate BALF. Therefore, we will increase the numbers of mice in each study, in order to have mice randomly designated to groups to collect BALF. We also plan to examine the relationship between SIRT1 and CLOCK genes, including the SIRT1/CLOCK/BMAL axis. Previous studies have shown that SIRT1 influences CLOCK genes directly, and that CS-exposure significantly impacts the expression of CLOCK genes via SIRT1/CLOCK/BMAL axis. We are interested in investigated the role of CS-exposure further in this process, with regard to SIRT1 enzymatic activity specifically, as well as how dietary intervention can alter any change that may occur. This will provide further understand of SIRT1 role in
CS-exposure alteration of CLOCK genes and circadian rhythm, as well as be the first study of its kind to demonstrate how dietary factors play a role in the alteration of a response by CLOCK in CS-exposed mouse model.