INTERACTIONS AMONG OZONE, OXYSTEROLS, AND ADVERSE HEALTH EFFECTS IN THE HUMAN AIRWAY

Adam Martin Speen

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Toxicology in the School of Medicine.

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Approved by

Ilona Jaspers

Michelle L. Hernandez

James M. Samet

Michael B. Fessler

Samir N. Kelada

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ABSTRACT

Adam Martin Speen: Interactions among Ozone, Oxysterols, and Adverse Health Effects in the Human Airway
(Under the direction of Ilona Jaspers)

Despite the wealth of studies examining the adverse health effects of ozone exposure and its association with increased airway inflammation, the mechanisms behind it have yet to be fully described. Ours and previous studies have shown that exposure to O_3 results in the formation of electrophilic lipid peroxidation products, notably through the oxidation of cholesterol in the airway lining fluid. These electrophilic oxysterols are capable of forming covalent linkages with nucleophilic centers of proteins, particularly lysine residues, thus altering cellular signaling pathways. Much remains to be learned about the identity of the reactive species and the range of potential proteins modified by the oxysterols in the lung and the biological consequences. Using 2D LC-MS/MS shotgun proteomics and "click" chemistry, we have generated a database of oxysterol-adducted proteins in airway epithelial cells treated with purified oxysterol Secosterol A as well as O_3-derived oxysterols. Among those oxysterol-adducted proteins were LXR and NLRP2. O_3-derived oxysterol adduction of LXR inhibits transcription of cholesterol transport and binding proteins and increases NF-κB activation and inflammatory cytokine signaling. NLRP2 is the most abundantly expressed member of the NLR family of proteins in human airway epithelial cells. Further, we show that exposure of epithelial cells to O_3 increases NLRP2 expression, active caspase-1 levels, and markers of inflammasome assembly, suggesting that NLRP2 inflammasome complexes play important roles in airway epithelial cells and more specifically in the context of O_3 exposure and oxysterol formation. Finally, we identify that the widely prescribed anti-psychotic drug Aripiprazole (APZ) has the potential to increase inflammatory response in human airway epithelial cells. We determine that APZ acts as an inhibitor of dehydrocholestrol reductase 7, resulting in an increased cellular concentration of the cholesterol precursor 7-dehydrocholesterol (7DHC). Ozonization of 7-DHC produces highly reactive oxysterol species in comparison to cholesterol ozonization products and our data shows that ozonization of 7-DHC compared to cholesterol results in increased inflammatory cytokine expression. Potentiation of the O_3 inflammatory response by APZ reveals a new paradigm of study and highlights the need for further drug-x-environment interaction research.

I dedicate my dissertation to my family. To my wife and best friend Abigail Browning whose love has supported me through my graduate school career. There is no one else I would rather laugh and cry with for the rest of my life. To my dad, who supported my non-linear career paths and has taught me that comfort should sometimes be sacrificed for fun and accomplishment. I am ever more driven to advance human health research because of his encouragement. To my mom, the teacher and scientist who's insatiable desire to gain and share knowledge has always inspired me to keep learning and keep teaching. Thank you.

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It takes a village to raise a child but it takes much more to raise a Ph.D. student. Foremost I would like to thank Dr. Ilona Jaspers and her entire laboratory, members past and present, for providing me with an outstanding environment in which to hone my technical and analytical skills as a biomedical researcher over the last five years. Ilona's commitment to excellence in all its facets is striking and her encouragement to be a better scientist and person resonates through both my professional and personal life. Her leadership shaped the type of people working in her lab, fostering a combination of personal responsibility, support, and fun that I have rarely experienced anywhere else in my life. Past doctoral students Rebecca Bauer and Megan Meyer paved the way for my research and abilities in Dr. Jaspers Lab. Postdoctoral fellows Meghan Rebuli, Shannon Jones, Erica Pawlak and Ellen Baker and fellow lab-mates Yael Escobar and Alexia Perryman pioneered new paths to help me on my research and normalized the graduate school life. Jessica R. Hoffman, undergraduate researcher extraordinaire, thank you for your tireless work and continued dedication to scientific research, I hope the best for you and your own doctoral career. Finally to the 2013 Tox Cohort, the best group that may ever grace the curriculum. Phil, Liz, Katelyn, Kim and Mimi; thanks for the trivia, laughs and drinks.

I would like to thank the technical and motivational support from Dr. Ned Porter and Hye-Young Kim at Vanderbilt University. Without their expertise in cholesterol chemistry and analytical techniques much of the progress my research represents would not have been possible. I am grateful to the two weeks of training I spent under your supervision in November 2016.

The Center for Environmental Medicine, Asthma, and Lung Biology (CEMALB) located in the EPA Human Studies Facility provided a rich clinical research environment in which to train. I received much support from the research coordinators, staff, and investigators who helped shape and implement my research strategies. The EPA NHEERL facilities greatly advanced my research through the utilization of their monitored and maintained ozone exposure chambers.

My committee of diverse researchers for UNC, EPA, and NIEHS have greatly informed my research designs and kept me on track during my graduate school career. I thank Drs. Michelle Hernandez, Samir Kelada, Michael Fessler, and Jim Samet for agreeing to serve on my committee and for helping to guide my graduate studies.

I also thank the funding sources that have made my studies possible. Dr. Jaspers R21-ES024666 the toxicology training grant T32-ES007126 and Big Data to Knowledge Training Program T32-LM012420-03.

PREFACE

Parts of this work were done in collaboration with other talented scientists and their efforts must be enumerated thusly:

Chapter 1 represents unpublished text to be adapted into a future review publication. I contrived of and wrote the main text and it was reviewed and edited by Ilona Jaspers. Dr. Michael Fessler provided feedback and notes on the organization and focus of the text.

Chapter 2, with minor adaptations to conform to dissertation format, was reprinted from already published work from the following citation:

Speen AM, Kim HH, Bauer RN, Meyer M, Gowdy KM, Fessler MB, Duncan KE, Liu W, Porter NA, Jaspers I. Ozone-derived oxysterols affect liver X receptor (LXR) signaling: a potential role for lipid-protein adducts. J Biol Chem 2016;291:25192–25206

Permission to include the article in its entirety in a PhD dissertation was retained from the American Chemical Society (publisher of JBC).

Chapter 3 represents unpublished primary research that was designed and directed by myself under the supervision of Dr. Ilona Jaspers. Dr. Ned Porter and Hye-Young Kim at Vanderbilt University supplied the alkynyl-tagged oxysterol probes, conducted the LC/MS/MS analysis, some of the click chemistry reactions, and consulted on the direction and design of this research. Jessica Hoffman performed various laboratory assays including qPCR and ELISA analysis. Phillip Clapp provided tissues and assisted with the immunohistochemistry protocols.

Chapter 4 represents original unpublished research by myself and undergraduate researcher Jessica R. Hoffman. I designed and implemented the study, however, under my supervision, Ms. Hoffman conducted much of the analysis and I adapted and edited parts of this chapter from her senior honor’s thesis. This manuscript will represent a co-first authored publication by myself and Ms. Hoffman in the future. Sterol measurements and adductomics analysis were conducted by Dr. Porter’s group at Vanderbilt University.

Chapter 5 represents original writing by myself, Adam Speen, as a summation and conclusion of the work detailed in the earlier chapters of this dissertation. I was the sole author of the writing included in this chapter with edits by Dr. Ilona Jaspers.

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TABLE OF CONTENTS

LIST OF TABLES xiv

LIST OF FIGURES xv

LIST OF ABBREVIATIONS xvii

CHAPTER 1: THE DUAL NATURE OF OXYSTEROLS IN THE HUMAN BODY 1

- 1.1 Introduction 1
  - 1.1.1 Cholesterol and Oxysterols in the Human Body. 1
  - 1.1.2 Distinguishing Oxysterols by Function and Route of Formation 3
- 1.2 Enzymatically Derived Oxysterols 5
  - 1.2.1 Enzyme Driven Oxysterol Formation 5
  - 1.2.2 Oxysterol Synthesis in the Biliary System 8
  - 1.2.3 Oxysterol Governed Cholesterol Homeostasis 8
  - 1.2.4 Oxysterols in Neuronal Signaling 9
  - 1.2.5 Oxysterol Signaling and Immune Function 9
- 1.3 Free-Radical Induced Oxysterols 10
  - 1.3.1 Common Synthesis of Free-Radical Induced Oxysterols 10
  - 1.3.2 Autoxidation and Mitochondrial Induced Free-Radical Oxysterol Signaling 11
- 1.4 Environmentally-Induced Oxysterols 12
  - 1.4.1 A New Classification: Exogenous Stressors and Oxysterol formation 12
  - 1.4.2 Ozone-derived Oxysterols 12
  - 1.4.3 Pathogens, Infection and Oxysterol Formation 13

1.4.4 Pharmaceutical Impact on Oxysterol Formation 14

1.5 Disease States and Oxysterols 15

1.5.1 Genetic Disorders affecting Oxysterol Synthesis 15

1.5.2 Oxysterols in Atherosclerosis 17

1.6 Scope and Impact: Oxysterols in the Human Airway 18

1.6.1 Airway Lining Fluid as a Target for Ozonization 18

1.6.2 Oxysterol Potential for Protein Adduction 20

1.6.3 Drug-x-Environment Interaction: Impact on Oxysterol Synthesis 21

1.6.4 Specific Aims of this Study and Overall Impact 22

REFERENCES 24

CHAPTER 2. – OZONE-DERIVED OXYSTEROLS AFFECT LIVER X RECEPTOR (LXR) SIGNALING: A POTENTIAL ROLE FOR LIPID-PROTEIN ADDUCTS. 35

2.1 Overview 35

2.2 Introduction 36

2.3 Materials and Methods 38

2.4 Results 47

2.5 Discussion 51

2.6 Figures 58

REFERENCES 67

CHAPTER 3: OZONE-DERIVED OXYSTEROL-PROTEIN ADDUCTS MODIFY NLRP2 INAIRWAY EPITHELIAL CELLS 74

3.1 Overview 74

3.2 Introduction 75

3.3 Materials and Methods 78

3.4 Results 85

3.5 Discussion ... 91

3.6 Figures: ... 97

REFERENCES ... 109

CHAPTER 4: THE SMALL MOLECULE ANTIPSYCHOTIC ARIPIPRAZOLE POTENTIATES OZONE-INDUCED INFLAMMATION IN AIRWAY EPITHELIUM ... 114

4.1 Overview ... 114

4.2 Introduction ... 115

4.3 Materials and Methods ... 117

4.4 Results ... 120

4.5 Discussion ... 123

4.6 Figures ... 129

REFERENCES ... 138

CHAPTER 5: IMPACT, NEW KNOWLEDGE GAPS, AND FUTURE STUDIES ... 142

5.1 O_3 Disruption of Sterol Homeostasis ... 142

5.1.1 Key Findings ... 142

5.1.2 Emergent Knowledge Gaps ... 143

5.1.3 Future Studies ... 144

5.2 Oxysterols: A Piece of the O_3-Induced Inflammation Puzzle ... 145

5.2.1 Key Findings ... 145

5.2.2 Emergent Knowledge Gaps ... 146

5.2.3 Future Studies ... 147

5.3 Novel Target: NLRP2 in Airway Epithelial Cells ... 148

5.3.1 Key Findings ... 148

5.3.2 Emergent Knowledge Gap ... 149

5.3.3 Future Studies 149
5.4 Intrinsic and Extrinsic Factors Determine O_3 Exposure Response.................................. 150
5.4.1 Key Findings.. 150
5.4.2 Emergent Knowledge Gaps 151
5.4.3 Future Studies 152
5.5 Overall Impact 154
REFERENCES 156

LIST OF TABLES

Table 3.1 Distinctive SecoA adducted 16HBE proteins 102

Table 4.1 APZ upregulated genes after O_3 exposure 134

Table 4.2 APZ downregulated genes after O_3 exposure 135

Table 4.3 DHCR7 Inhibiting Pharmaceutical Drugs 137

LIST OF FIGURES

Figure 1.1 Chemical Structure of Sterols and Cholesterol 2

Figure 1.2 Side-Chain Modification of Commonly Formed Oxysterols 3

Figure 1.3 Three Classifications of Oxysterol Formation 5

Figure 1.4 Cholesterol Biosynthesis Pathway and Generation of Oxysterols Necessary for Downstream Signaling and Sterol Homeostasis........................... 6

Figure 1.5 Oxysterol Activated LXR Signaling 7

Figure 1.6 Cholesterol Ozonization Products 13

Figure 1.7 Constituents of the Airway Surface Liquid 19

Figure 2.1: Cholesterol and the major oxysterols formed in the reaction with ozone. 58

Figure 2.2: Oxysterol concentrations measured in cells and apical washes exposed to filtered air or O_3 and their HPLC-MS profiles. 59

Figure 2.3: Oxysterol concentrations measured in airway BALF 60

Figure 2.4: Cholesterol efflux pump protein and pro-inflammatory cytokines gene expression levels in 16HBE cells. 61

Figure 2.5: IL-6 expression and production in the lung of O_3 exposed LXRα deficient mice.. 62

Figure 2.6 Simplified steps of protein catch and photo-release using a-Seco A probe in 16 HBE cells. 63

Figure 2.7: Protein adduct formation is observable in 20 µM a-Chol supplemented 16HBE cells exposed to O_3.. 64

Figure 2.8: Seco A altered cholesterol efflux pump gene expression when treated with Seco A and the T09 LXR agonist in sequence. 65

Figure 2.9: Oxysterol altered inflammatory signaling 66

Figure 3.1: O_3-derived oxysterol protein adduction 97

Figure 3.2: Click Chemistry and LC/MS Analysis 98

Figure 3.3: Structure of an *a*-SecoA adduct on a protein lysine after photo-release 99

Figure 3.4: NLRP2 (NACHT, LRR and PYD domains-containing Protein 2) modification with *a*-SecoA 100

Figure 3.5: SecoA Adductome Protein Association Network 101

Figure 3.6: Relative abundance of NLR family protein and mRNA in human airway epithelial cells........ 103

Figure 3.7: Protein catch and photo-release using a-Seco A and *a*-cholesterol in 16 HBE and THP-1 cells........ 104

Figure 3.8: Immunohistochemistry and immunoblot analysis of O_3 induced levels of NLRP2 and inflammasome activity in human airway epithelial cells........ 105

Figure 3.9: shRNA knockdown of NLRP2 in 16HBE cells 106

Figure 3.10: NLRP2 shRNA inhibits O_3-induced inflammatory signaling 107

Figure 3.11: Anakinra and Cas-1 inhibitors also inhibit inflammatory signaling in 16HBE cells. 108

Figure 4.1: APZ inhibition of cholesterol synthesis pathway, 7-DHC and cholesterol structures........ 129

Figure 4.2: APZ and AY9944 effects on 7-DHC levels in airway epithelial cells........ 130

Figure 4.3: APZ potentiates O_3-induced *IL-6* and *IL-8* expression 131

Figure 4.4. Stimulation with TNFα does not incite APZ-induced *IL-6* and *IL-8* expression. 132

Figure 4.5. Nanostring analysis of gene expression in 16HBE cells treated with APZ 133

Figure 4.6: Ozonized 7-DHC increases *IL-6* and *IL-8* expression to a greater extent than ozonized cholesterol 136

LIST OF ABBREVIATIONS

4-HNE – 4-Hydroxynonenal

7-DHC – 7-dehydrocholesterol

a-Chol – alkynyl Cholesterol

a-SecoA – alkynyl Secosterol A

ABCA1 – ATP binding cassette transporter A1

ABCG1 – ATP binding cassette transporter G1

ALI – Air-Liquid Interface

APZ – Aripiprazole

ASC – Apoptosis-associated speck-like protein containing a CARD

ASL – Airway Surface Liquid

BALF – Bronchoalveolar Lavage Fluid

Cas-1 – Caspase 1

cDNA – Complementary DNA

CNS – Central Nervous System

CYP – Cytochrome P450

DAMP – Damage Associated Molecular Pattern

DHCR7 – Dehydrocholesterol reductase 7

DMSO – Dimethyl sulfoxide

EC – Epithelial Cell

ELISA – Enzyme-linked immunosorbent assay

ER – Endoplasmic Reticulum

FASB – Fatty Acid Synthase beta subunit

FBS – Fetal Bovine Serum

FCH – Familial Combined Hyperlipidemia

GPR – G-protein coupled receptor

GSTM1 – Glutathione S-transferase Mu 1

HDL – High Density Lipoprotein

HIS3 – Histone 3

HMG-CoA – 3-hydroxy-3-methyl-glutaryl-coenzyme A

IL-1β – Interleukin-1β

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-18 – Interleukin 18

Insig – Insulin induced gene

LC-MS/MS – Tandem Mass Spectrometry

LDH – Lactate Dehydrogenase

LDL – Low-density lipoprotein

LOP – Lipid oxidation product

LXR – Liver X Receptor

NAAQS – National Ambient Air Quality Standards

NLRP – NACHT, LRR and PYD domains-containing protein

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

MEM – Minimal Essential Medium

O_3 – Ozone

Oxysterol – Oxidized cholesterol

PAMP – Pathogen Associated Molecular Pattern

PCR – Polymerase Chain Reaction

PYCARD – PYD and CARD domain containing

qRT-PCR – Quantitative Real Time PCR

ROS – Reactive Oxygen Species

SCAP – SREBP cleavage-activating protein

Seco A – Secosterol A

Seco B – Secosterol B

SLOS – Smith Lemli Optiz Syndrome

SREBP – Sterol regulatory element binding protein

ROS – Reactive oxygen species

RXR – Retinoid X Receptor

TNF – Tumor necrosis factor

TZD - Trazodone

VOCs – Volatile organic compounds

WT – Wild Type

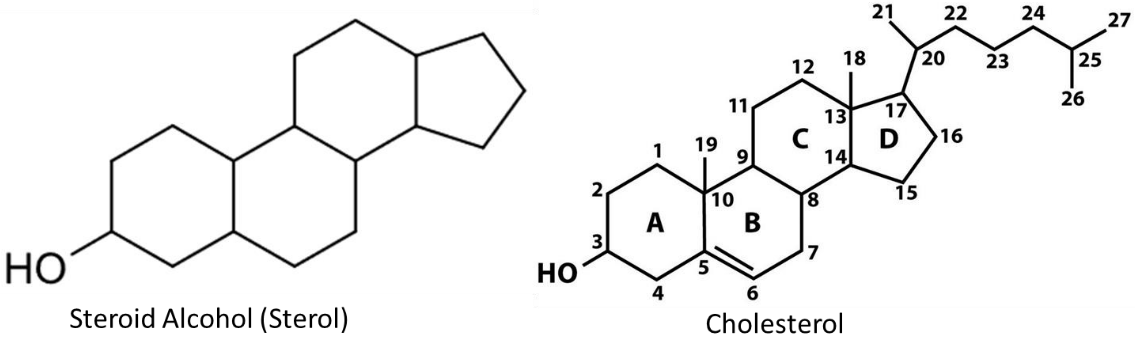
CHAPTER 1: THE DUAL NATURE OF OXYSTEROLS IN THE HUMAN BODY

1.1 Introduction

1.1.1 Cholesterol and Oxysterols in the Human Body.

Medical biologists and toxicologists have expressed interest in oxysterol biochemistry in the human body for at least the last 50 years, however, only recently have they been studied as a potential target for interaction with environmental stressors and as a marker of health in various tissues. The aim of my dissertation as a whole is to present a novel understanding of oxysterol formation and their biochemical properties, providing new information to researchers studying the impact of cholesterol and cholesterol modification by environmental exposures in human health. The purpose of this chapter is to introduce the reader to the function of oxysterols in the human body, distinguish differences in routes of oxysterol formation, and discuss their potential for adverse health effects. Principally, it is important to place our discussion of oxysterols in the context of environmental pollutant exposures and the human airway epithelium, the tissue of interest and focus of the primary research findings discussed in the later chapters.

Steroid alcohols (Figure 1.1), or sterols are organic molecules with a polar hydroxyl group at the 3-position of the A-ring and are formed through the HMG-CoA reductase pathway (1-3). In humans, cholesterol is the most abundant and well-studied sterol. In general, sterols are necessary for key metabolic functions in normal cellular activities, most notably maintaining cellular membrane integrity, waste excretion, and the formation of signaling molecules like steroids (4). Thus, sterol concentration is tightly regulated by the human body. Sterol precursor molecules as well as modified cholesterol have been associated with cholesterol homeostasis and transport, likely since the terpenoids and hopanoids of early bacteria (5,6). Early modified sterol messengers were produced through nonenzymatic forces and served to induce cellular cholesterol management, waste excretion, and transcription signaling (7,8). Sterol modification has been evolutionarily preserved as an integral process necessary for survival in a variety of species, including mammals.



Steroid Alcohol (Sterol)

Cholesterol

Figure 1.1: Chemical Structure of Sterols and Cholesterol

Oxidized cholesterols (oxysterols) are a form of modified sterols, which are known to direct biological functions like those mentioned previously. Measured concentrations of oxysterols in the human body vary depending on the cellular tissue, metabolic capacity, and health of the individual (9,10). For example, the plasma of smokers exhibits higher levels of the oxysterol 7β-hydroxy-cholesterol compared to nonsmokers (11,12). Under healthy conditions, oxysterols typically exist in trace amounts, at approximately 1000-fold less than cholesterol, however, their distribution throughout the individual cell and human body is not uniform and an increase in the oxysterol/cholesterol ratio can lead to pathophysiological outcomes (13,14). In order to better understand the adverse outcomes associated with oxysterols in the human body it is important to discuss how they are formed and their primary function.

1.1.2 Distinguishing Oxysterols by Function and Route of Formation

When writing about oxysterol in the human body, route of formation and function need to be considered, however, making these distinctions quickly becomes increasingly complex. Primarily, the species of oxysterol formed depends on the precursor sterol that is oxidized. Depending on the sterol target, primary oxysterols exhibit either a side chain or ring modified oxysterol while secondary oxysterols can have multiple modifications (15). Through varied modifications there are a great variety of oxysterol species capable of being formed in the human body. The complexity intensifies as oxysterol species formation can be determined by the pH of the environment in which it is formed and the capacity for primary oxysterols to be quickly metabolized and transformed into secondary structures (16). To varying degrees, most oxysterols are electrophilic, in that they have the capacity to accept electrons and illicit cellular responses by bonding to another compound (17). Their electrophilic nature allows oxysterols to both act as key signaling molecules in the body and act as potentially damaging compounds with the capacity to bind to nucleic acids and proteins. As a consequence of the complexity governing what is currently known about oxysterols in the human body, it is the best practice to make delineations for oxysterol species based on route of formation.

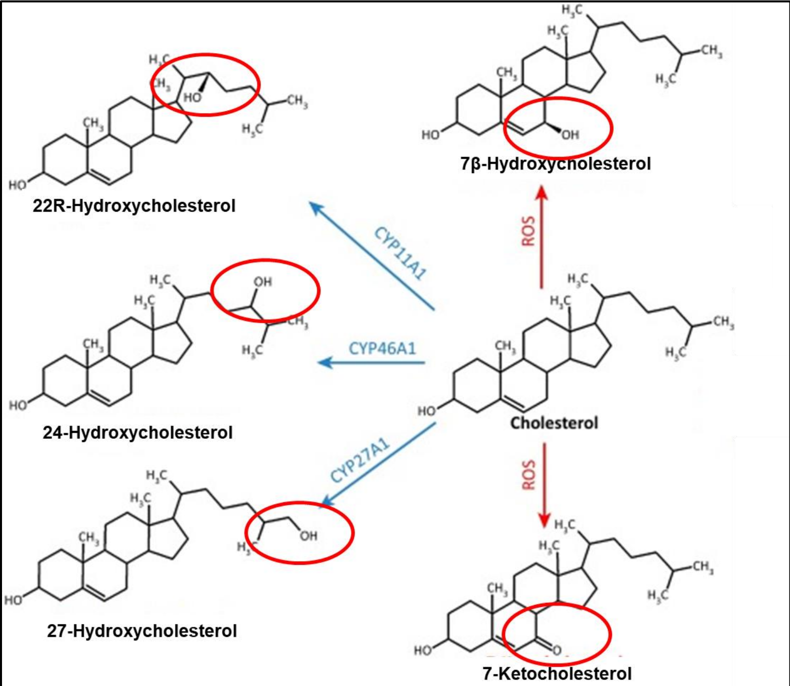


Figure 1.2: Side-Chain Modification of Commonly Formed Oxysterols

Traditionally, oxysterols in the human body are split into two groups, enzymatically derived and free-radical derived (18,19). While the majority of oxysterols are created enzymatically through cytochrome P450 (CYP) hydrolysis, many unique oxysterol species are formed through autoxidation and other non-enzymatic metabolism and alteration. With exceptions, enzymatically derived oxysterols typically exhibit modification to the cholesterol side chain, while free-radical derived oxysterols generally exhibit modification to the cholesterol ring 5,6 double bond position, forming aldehyde, epoxy, and keto groups capable of electrophilic attack on various cellular components (Figure 1.2) (20,21). The existence of this division in the characterization of oxysterols has led many researchers to split oxysterols into two groups; those involved in cellular signaling necessary for normal cell function or oxysterols formed as metabolic products of cellular waste capable of cellular damage (15,22). Despite the number of reviews noting these two groups, this dichotomy is problematic as even many free-radical derived oxysterols are necessary for normal cellular processes and cannot be simply described as causative of adverse effects. The potential for individual oxysterol species to cause harm is better determined by examining the route of oxysterol formation (i.e. which oxidizing agent created them) and their concentration in a certain tissue. Thus, a more nuanced examination of oxysterols and a third category may be necessary.

For the purposes of this review we will divide oxysterols into three separate categories: 1) enzyme-induced, 2) free-radical-induced, and 3) environmentally-induced. Both enzyme-induced and free radical-induced occur naturally through normal cellular activity (Figure 1.3). In contrast, environmentally-induced oxysterols, are formed due to exogenous stressors and pose a unique challenge to cellular function and viability. Further, in each group we will discuss the biological systems influenced by the prominent oxysterol species formed and any potential for adverse health effects. Through enumerating these divisions, we will examine the differences in formation, structure, and action of the species formed including their association with specific disease states. We pay particular attention to the third group of environmentally-induced oxysterols as they represent unique species with highly reactive electrophilic modifications to the sterol ring structure and until now have not been studied.

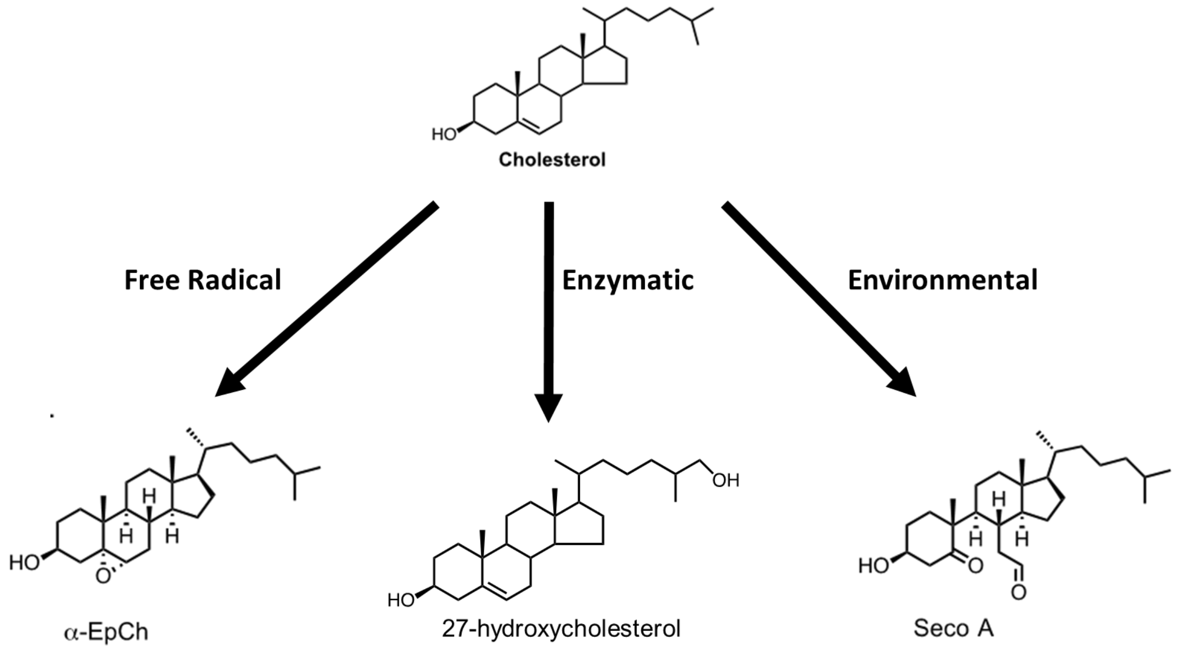


Figure 1.3: Three Classifications of Oxysterol Formation

1.2 Enzymatically Derived Oxysterols

1.2.1 Enzyme Driven Oxysterol Formation

The majority of normal oxysterols formed in the human body are a product of enzymatic conversion, driven primarily by CYP enzymes (23). Oxygenases such as CYPs serve to add an oxygen group onto cholesterol or its sterol precursors, forming oxysterols. After oxysterol formation, the addition of a single oxidation reaction reduces an oxysterol’s half-life and directs the molecule to be degraded and excreted (24,25). Conversion of cholesterol into bile acids, steroid hormones, and signaling molecules, however, requires several oxidation steps and multiple enzymatic conversions (25). Enzymatically-derived oxysterols are strictly regulated and the more prominent species serve to maintain cholesterol homeostasis, waste removal, and cell developmental processes. Although some oxysterols are formed due to the action of a single enzyme, many are generated by the action of multiple enzymes. For example, the oxysterol 25-hydroxycholesterol can be generated by CYP27A1, CYP46A1, or CYP3A4, making tracing its origin difficult (26). Here, we highlight the function of some of the more prominent oxysterol species formed due to enzymatic oxidation.

Shown in Figure 1.4, the oxysterol 24,25-epoxycholesterol is a special case as it is formed by oxidation of a precursor sterol species formed early in the cholesterol synthesis pathway by CYP46A1. 24,25-epoxycholesterol can be formed in one of two ways: 1) as a *de novo* synthesis shunt of the normal cholesterol synthetic mevalonate pathway (27,28) or 2)

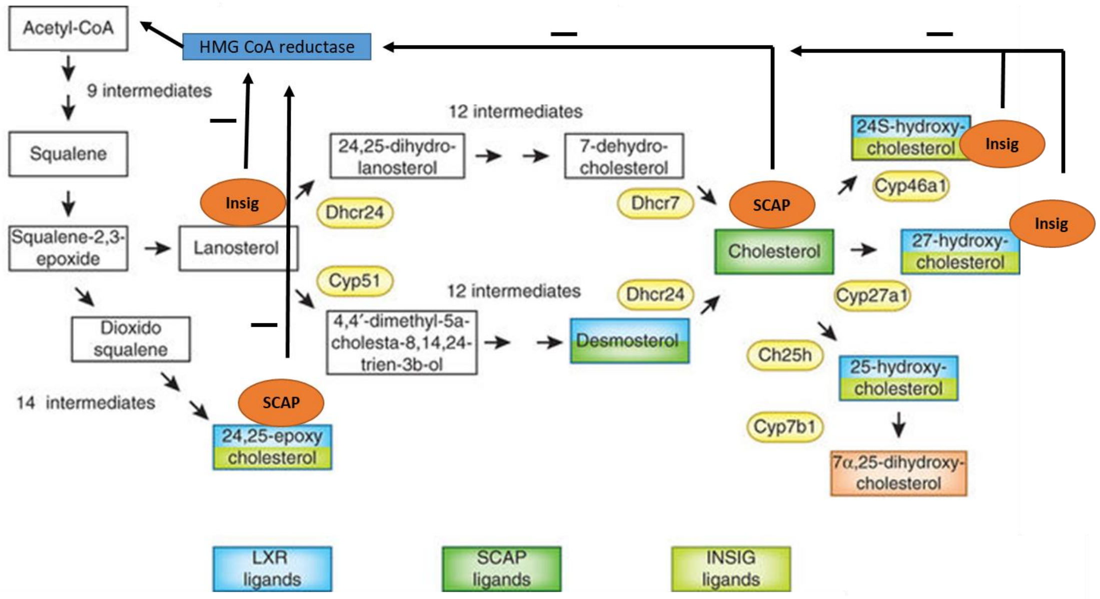

Acetyl-CoA
HMG CoA reductase
9 intermediates
12 intermediates
Squalene
24,25-dihydro-lanosterol
7-dehydro-cholesterol
24S-hydroxy-cholesterol
Insig
Insig
Dhcr24
Dhcr7
SCAP
Cyp46a1
Squalene-2,3-epoxide
Lanosterol
Cholesterol
27-hydroxy-cholesterol
Insig
Cyp51
12 intermediates
Dhcr24
Cyp27a1
Dioxido squalene
4,4′-dimethyl-5a-cholesta-8,14,24-trien-3b-ol
Desmosterol
Ch25h
25-hydroxy-cholesterol
14 intermediates
SCAP
24,25-epoxy cholesterol
Cyp7b1
7α,25-dihydroxy-cholesterol
LXR ligands
SCAP ligands
INSIG ligands

Figure 1.4: Cholesterol Biosynthesis Pathway and Generation of Oxysterols Necessary for Downstream Signaling and Sterol Homeostasis. Adapted from Spann et. al 2013

through enzymatic oxidation of desmosterol. In both cases 24,25-epoxycholesterol serves to suppress 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) activity and increase cholesterol transport gene expression, acting as a feedback defense mechanism to prevent excess synthesis of new cholesterol (29).

In addition to regulation over cholesterol synthesis, a variety of metabolically formed oxysterols are responsible for regulation of total sterol content in both the cell and the human body. Cholesterol homeostasis signaling occurs primarily through the Liver X Receptor (LXR) transcription pathway. LXR, and its heterodimer the Retinoic X Receptor (RXR) are responsible for the expression of cholesterol efflux and binding proteins including ATP Binding Cassette Transporter Proteins A1 and G1 (ABCA1 and ABCG1), Fatty Acid Synthase B (FASB), and SREBP. A variety of CYP27A1 side chain modified oxysterols such as 22-R-hydroxycholesterol are capable of binding to the LXR/RXR heterodimer, signaling the activation of cholesterol transport and binding proteins mentioned above and shunting resources towards the conversion of excess cholesterol into bile waste (Figure 1.5) (30,31).

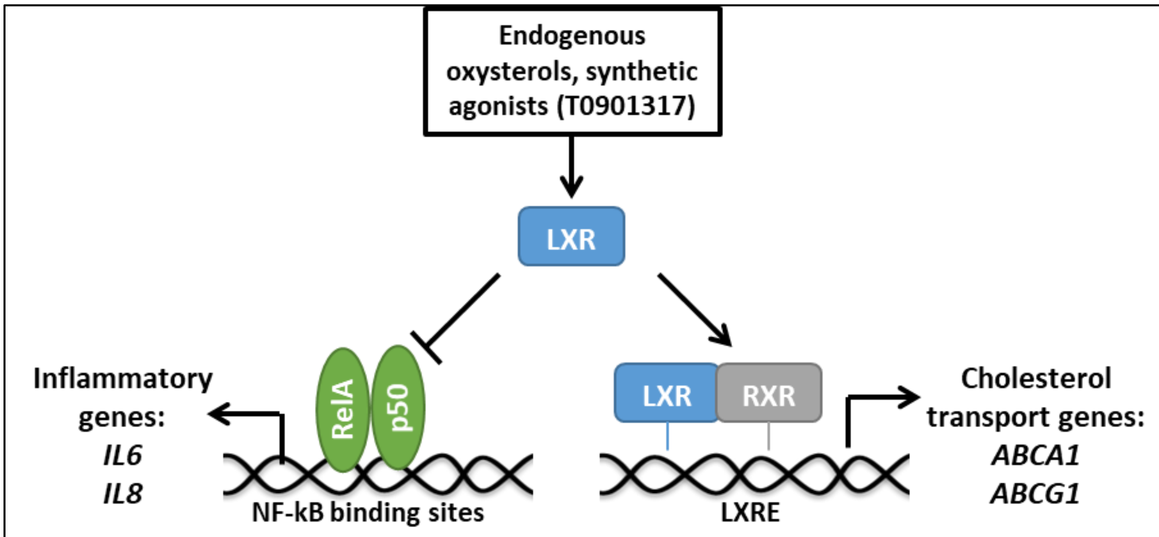

Endogenous oxysterols, synthetic agonists (T0901317)
LXR
Inflammatory genes:
IL6
IL8
RelA
p50
NF-kB binding sites
LXR
RXR
LXRE
Cholesterol transport genes:
ABCA1
ABCG1

Figure 1.5: Oxysterol Activated LXR Signaling

1.2.2 Oxysterol Synthesis in the Biliary System

Oxysterol synthesis is of concern in the hepatobiliary system and governs the constituents and ratio of lipid content in bile acid for excretion (23,32,33). The major metabolic and rate limiting enzymatic reaction of biliary acid synthesis in humans starts with CYP7A1, cholesterol 7α-hydroxylase, metabolism of cholesterol to form 7α-hydroxycholesterol (23,24,34-36). Alternatively, the oxysterol 27-hydroxycholesterol is formed through CYP27A1, 27-hydroxylase, in the mitochondrial cytoplasm and functions with two cofactor proteins andrenodoxin and andrenodoxin reductase to hydroxylate a variety of sterols at the 27 position leading to the formation of cholestenoic acid (24). Current research indicates that other oxysterols such as 24S-hydroxycholesterol in the brain also contribute to bile acid synthesis and excretion and is discussed briefly in the following section. Overall the majority of studies indicate that oxysterol species involved in bile acid formation are exceptionally lipophilic and easily enter the biliary system as waste for excretion.

1.2.3 Oxysterol Governed Cholesterol Homeostasis

Principally, sterols and oxysterols act as sensor molecules, providing feedback regulation to govern total cholesterol content in the cell or whole tissue. Oxysterols exert posttranscriptional regulation of cholesterol homeostasis by controlling sterol transport throughout the cell and inhibiting *de novo* cholesterol synthesis as noted in Figure 1.4 (37). The oxysterol 25-hydroxycholesterol mentioned above, promotes the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate-limiting enzyme in cholesterol synthesis by accelerating ubiquitination of HMGCR (38,39). Endoplasmic Reticulum (ER) transmembrane insulin induced proteins (Insig-1 and Insig-2) bind to 25-hydroxycholesterol, allowing for greater interaction with the sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP), which allows for precise sterol sensing within the cell and inhibits further cholesterol synthesis and oxysterol formation (40,41). Cholesterol-derived oxysterol, 24,25-dihydrolanosterol, formed when CYP51 binds to Insig proteins, which in turn binds to the HMGCR, ubiquitinating the protein for proteasomal degradation and slowed cholesterol synthesis (15,42).

1.2.4 Oxysterols in Neuronal Signaling

As mentioned above, in addition to hepatobiliary excretion, oxysterols serve as potent signaling molecule and waste product in various tissues in the human body. The brain and central nervous system (CNS) contains some of the most lipid and cholesterol rich cells and metabolism of cholesterol is a key component of normal function and removal of waste products from the CNS infrastructure. The most abundant oxysterol in the brain, 24S-hyroxycholesterol is formed by metabolism of cholesterol by CYP46A1 (24-hydroxylase) in neuronal cells (43). 24S-hydroxycholesterol readily passes through the blood brain barrier, activates reverse cholesterol transport signaling by activating LXR transcription pathway and increasing cholesterol efflux from cells in the CNS. Sterol waste product removal from the CNS and many tissues is very important in maintaining optimal cellular health and deficiencies in the process of enzyme-derived oxysterol formation can be associated with the propagation of neurodegenerative disorders and other adverse human health conditions (44,45).

1.2.5 Oxysterol Signaling and Immune Function

Oxysterol control over the fundamental cellular processes discussed above applies to many systems governing human health and crucial attention must be paid to their involvement in the context of immune cell development and immune response. Cholesterol research has pointed to a connection between optimum cholesterol homeostasis and host defense against pathogens and inflammation by regulating the cell membrane barrier integrity and decreasing waste generated oxidant stress (46). The oxysterol activation of LXR, discussed above and shown in Figure 1.5, has been repeatedly shown to also have a transrepressive effect on NF-kB pro-inflammatory gene induction (47-49) and mitigates immune response to inflammatory stimuli. Studies have also determined that immune T-cell proliferation and activation is sterol dependent through LXR and cholesterol transport signaling (50,51). In addition to oxysterol feedback on cholesterol homeostasis governing immune cell proliferation, oxysterols can directly bind to immune receptors and alter immune response. The oxysterol 7α,25-dihydroxycholesterol has been shown to be a potent agonist for G protein coupled receptor 183 (GPR183), governing B cell maturation in the lymph nodes and T cell activity (52,53). Moreover, a deficiency in CYP7B1 and decreased 7α,25-dihydroxycholesterol results in deficient T cell and B cell immune response (54). Furthermore, macrophage and dendritic cell migration, differentiation, and phagocytic capacity are impaired by cholesterol dysregulation and increased oxysterol concentrations (55,56).

1.3 Free-Radical Induced Oxysterols

1.3.1 Common Synthesis of Free-Radical Induced Oxysterols

Nonenzymatic, or free-radical induced oxysterol formation occurs through cholesterol or precursor sterol species interaction with lipid peroxides, transition metal ions and reactive oxygen species (ROS) present throughout the human body (20). The random character of free radical oxidation and automated rearrangement of these reactive species confounds the exact mechanism and route of formation for individual oxysterol species. Typically, oxidation of cholesterol resembles a lipid peroxidation reaction by abstracting the hydrogen from the C-7 position, forming a radical carbon capable of further oxidation into cholesterol peroxyl radical and finally into relatively stable oxysterol species such as cholesterol hydroperoxide (Figure 1.1). The C-7 position is particularly susceptible to oxidation as the carbon-hydrogen bond has low dissociation energy and can be readily abstracted (57). Oxidation of cholesterol at the C-7 ring and formation of cholesterol hydroperoxide is short lived however, as it is further transformed into polar hydroxyl, keto, hydroperoxy, epoxy, or carboxyl moieties (58,59). Free-radical derived oxysterols such as these have unique interactions with normal cellular functions leading to adverse cellular events.

1.3.2 Autoxidation and Mitochondrial Induced Free-Radical Oxysterol Signaling

Free-radical formation through normal cellular activity is largely driven by mitochondrial respiration and the formation of ROS. The mitochondrial electron transport chain uses molecular oxygen molecules and creates superoxide anion radicals and subsequent breakdown products hydrogen peroxide and hydroxyl radicals. These reactive oxygen species (ROS) are involved in type 1 autoxidation of cholesterol and serve to typically modify the ring structure of cholesterol, forming some of the species mentioned above including 7β-hydroxy-cholesterol, 7-ketocholesterol, and 5α,6α-epoxycholesterol (18). Cellular health influences ROS generation through normal cellular respiration. The buildup of oxysterol autooxidation products can cause severe cytotoxic events in a cell. Excess 7-ketocholesterol and 27-hydroxycholesterol alter the biophysical property of the cell membrane, disrupting integrity and triggering apoptotic signaling (60-63).

1.4 Environmentally-Induced Oxysterols

1.4.1 A New Classification: Exogenous Stressors and Oxysterol formation

Despite the breadth of literature describing the mechanisms governing the metabolism and function of enzymatically-derived oxysterols, little focus has been given to environmentally-induced oxysterols. Environmental stressors capable of forming oxysterols include pollutants, pathogens, and xenobiotics. Like all oxysterols, environmentally derived oxysterols are formed through an oxidation reaction with cholesterol or its precursors, however, the resulting species can be unique and exhibit a heightened capacity for electrophilic attack leading to cellular dysfunction and death. For this reason, we will highlight a few known environmental stressors involved in the formation of oxysterols including Ozone (O_3).

1.4.2 Ozone-derived Oxysterols

The clearest example of environmentally-derived oxysterols are those formed due to ozonization. O_3 induced oxidation of cholesterol is a special case as a specific exogenous pollutant is capable of forming oxysterols inside the human lung. O_3 is a ubiquitous pollutant and 80% of the global population is exposed to levels exceeding WHO guidelines throughout the year. Moreover, the target organ for O_3 exposure, the lung, is lined with lipid rich surfactant made up of phospholipids, surfactant proteins and cholesterol. Cholesterol presents a prime target for ozonization, as it is the primary neutral lipid component of the airway lining fluid (64,65). It has been shown that the initial reaction of ozone with cholesterol yields 1,2,3-trioxolane, which undergoes rapid and spontaneous decomposition to yield several additional reactive entities. These intermediary oxysterols ultimately undergo cleavage of the C-5 – C-6 double bond forming stable ozone-derived oxysterols such as 6-oxo-3,5-diol, 3,5,6-triol, α-epoxycholesterol (EpChα), β-epoxycholesterol (EpChβ), Secosterol A (SecoA) and Secosterol B (SecoB) (66,67) (Figure 1.6). Studies examining electrophilic oxysterols, including our own, have shown the capacity for O_3-derived oxysterols to form adducts with a variety of protein targets including those associated with cholesterol homeostasis and inflammatory signaling (68). The integrity of the airway epithelium is reliant on consistent cholesterol composition and concentration and even modest changes to the sterol profile of the airway could compromise the surface tension-reducing properties of surfactant and impact respiratory health (64).

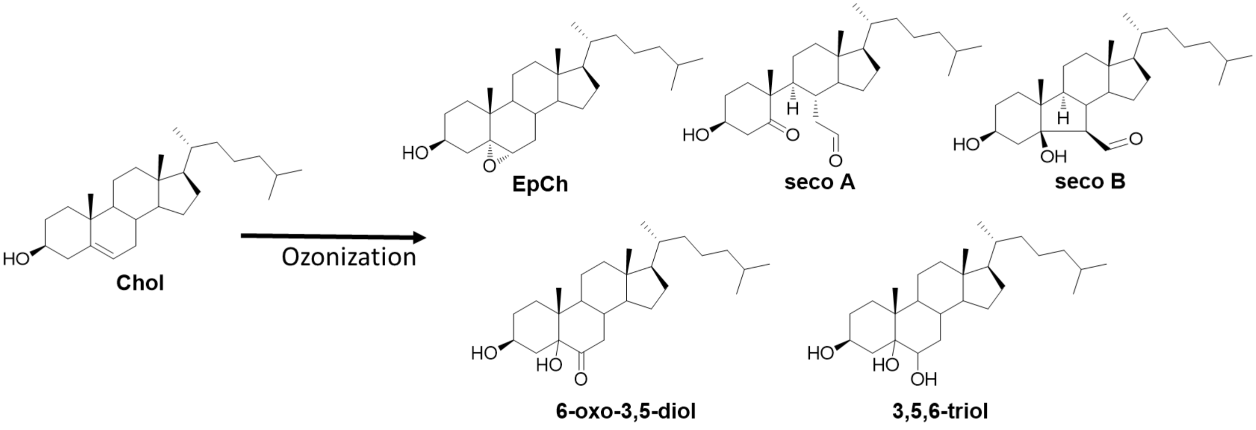


Figure 1.6: Cholesterol Ozonization Products

1.4.3 Pathogens, Infection and Oxysterol Formation

In addition to oxysterols formed due to native free-radicals, foreign bodies and pathogens can serve to form additional reactive species and drive the formation of oxysterols. Chronic hepatitis C virus infection has been shown to enhance concentrations of certain oxysterols in the serum of patients (69). 7α-hydroxycholesterol, 4β-hydroxycholesterol, and 25-hydroxycholesterol are all significantly elevated in individuals with chronic hepatitis C, suggesting that oxidative stress and inflammation caused by the infection influences systemic sterol levels (70). It is unknown whether this change in oxysterol concentration is associated with propagating the disease state or merely a significant marker of the infection. Nevertheless, it is interesting to consider how exogenous stressors can impact oxysterol formation in the human body and should be considered when examining health effects and biomarkers of disease.

1.4.4 Pharmaceutical Impact on Oxysterol Formation

Pharmaceutical safety testing research has indicated that a growing list of commonly used drugs have the capacity to modify oxysterol formation either by desaturating cholesterol or altering overall sterol concentrations in the human body. Although many pharmaceuticals were designed to modify enzymatic activity or alter the availability of cholesterol, we believe this constitutes an exogenous stressor influencing oxysterol homeostasis and alters susceptibility to human disease. Statins, one of the most widely prescribed classes of drugs in the world, are designed to reduce systemic cholesterol available by inhibiting HMG-CoA reductase. Although it has not been clinically shown, epidemiologic evidence demonstrates that statins may protect against pollutant induced injury, an effect possibly mediated by cholesterol availability (71). Alternatively, other common drugs have the ability to increase oxysterol presence causing cellular dysfunction. As an example, patients treated with antiepileptic drugs such as phenobarbital, carbamazepine or phenytoin exhibited 10-20 fold elevated levels of oxysterol 4β-hydroxycholesterol (4β-HC) (72,73). The increase in 4β-HC may be due to a drug induced increase of CYP3A4 activity, however, the subsequent presence of 4β-HC found in atherosclerotic plaques compared to other prominent oxysterol species has not been fully explained (73). Additionally, the primary research discussed in Chapter 4 of this dissertation discusses the potential for small molecule antipsychotics and antidepressants such as

Aripiprazole (APZ) and Trazodone (TZD) to inhibit cholesterol synthesis steps and lead to oxidized precursor sterol molecules and uniquely reactive oxysterols. Considering that environmental exposure may coincide with pharmaceutical action on cholesterol availability, the potential for interaction and synergistic adverse effects is great.

1.5 Disease States and Oxysterols

1.5.1 Genetic Disorders affecting Oxysterol Synthesis

Cholesterol precursor molecules are known to be even more prone to electrophilic attack than cholesterol itself and any genetic modifications to cholesterol synthesis could lead to increased oxysterol formation and detrimental health effects. The genetic disorder known as Smith-Lemli-Opitz syndrome (SLOS) provides a unique look at the adverse impact altered sterol availability can have on human health. Individuals suffering from SLOS experience decreased overall cholesterol levels and an increase in the 7-DHC to cholesterol ratio, developmental and cognitive impairments, poor epithelial integrity and increased basal inflammatory signaling as a result of dysregulated cholesterol synthesis (74,75). Genetic mutations of the 7-DHC reductase (DHCR7) gene are the hallmark of SLOS, which is characterized by high circulating levels of 7-dehydrochlesterol (7-DHC) and low serum cholesterol (76). In individuals with SLOS, the final step in cholesterol synthesis, involving the conversion of 7-DHC to cholesterol by DHCR7, is disrupted. The resulting increased levels of 7-DHC, which has the highest rate constant for the propagation of free radical peroxidation of any lipid studied to date, would be very sensitive to oxidative damage (77). UV sensitivity is a hallmark of SLOS patients, exacerbating dermal inflammation and cell death, so it is possible that oxidant pollutants like O_3 could have the same impact, especially in a cholesterol rich environment like the lung (78).

In addition to SLOS there are other disease states associated with dysregulation of oxysterols including dyslipidemia, atherosclerosis, and infection. Familial combined hyperlipidemia (FCH) is characterized by increased atherosclerotic lesions associated with oxidative damage to lipids circulating in the plasma (79). Oxidation of plasma cholesterol to 7-keto and 7β-hydroxycholesterol is elevated in these patients and has been shown to be correlated with propagation of increased systemic inflammation leading to cardiovascular disease and atherosclerosis (80).

Ancillary evidence has been presented regarding the role of oxysterols in neurodegenerative diseases. 24-hydrocholesterol is the principal oxysterol made by neuronal cells and is necessary for normal CNS function (81). Patients with Huntington's disease, however, exhibit severely decreased levels of 24-hydrocholesterol (82,83). 24-hydrocholesterol is normally associated with initiating reverse cholesterol synthesis and waste removal from CNS tissues, any disruption of this process would result in accumulation of cellular waste products and a decrease in cellular viability commonly associated with Huntington's Disease (43-45). Similar evidence has been presented regarding Alzheimer's disease and tau protein occlusions, however, conclusive clinical evidence has yet to be presented (84,85). Further, the use of dysregulated oxysterol formation and presence as a biomarker for neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) has been proposed (86). These examples show that cholesterol is an essential building block of the brain and a critical component of many biological functions, alteration of cholesterol synthesis or normal levels of oxysterols can result in cellular waste build up and debilitating disease states.

1.5.2 Oxysterols in Atherosclerosis

The role of oxysterols in atherosclerosis is of particular interest as one of the first steps of plaque formation, macrophage conversion to foam cells, involves a high uptake of oxidized LDL and an indiscriminant uptake of other oxidized lipids (87,88). Although high levels of oxysterols have been associated with the development of atherosclerotic plaques and lesions, little conclusive evidence has been reported as to the route of formation of the oxysterols present and the mechanism driving atherosclerotic plaque development (89,90).

Due to the associated progression of atherosclerosis most of the oxysterols present have been studied as a product of free-radical oxidation, however, their origin is not always clear. For example, elevated 27-hydroxycholesterol has been shown to promote atherosclerosis by altering sterol uptake in human macrophages and mouse atherosclerotic lesions, but little information is available as to why there is such an increase in 27-hydroxycholesterol availability and why build up in macrophages induced plaques (91,92).

In addition to free-radical induced oxysterols, 7a- and 7B-OH-Ch 5,6-epoxycholesterol, 7-oxo-cholesterol and Secosterols A and B all have been recorded at higher than normal concentrations in atherosclerotic lesions, but their source has not been elucidated. Interestingly, Secosterols A and B are thought to be exclusively oxysterol products specific to oxidation by ambient O_3, making their presence in atherosclerotic plaques cells perplexing (93). One report indicates that their presence in foam cells could be due to substantial flux of oxysterols through the airway and into circulation (94). The abnormal accumulation of macrophages in the lung during dyslipidemic events could be driving the oxysterol efflux out of the airway, however, current measurement techniques cannot identify oxysterol origin with complete certainty (95). The field of atherosclerosis could be well served by early and accurate analysis of the oxysterol species and concentrations found in early macrophage foam cells and plaques as it is still unclear whether reactive oxysterols have a causal role in atherogenesis.

1.6 Scope and Impact: Oxysterols in the Human Airway

Through varied function and formation in the human body, oxysterols have positioned themselves as a target of interest in biomedical and biochemical research. Under normal conditions, oxysterols are key to cellular function across the human body, thus any perturbation to their formation could be a cause for concern. In this review, we considered oxysterol formation through enzymatic and free-radical means, with the latter category split into those formed endogenously and those formed through external stressors. When examining those oxysterols formed due to genetic disorder, environmental stressors, drugs, and infection, there is overwhelmingly compelling evidence to explore oxysterols as both potential causative factors and biomarkers of disease states. The focus of our research lies in pollutant-induced inflammation and immune response and our primary research papers serve to uncover how the pollutant O_3 impacts oxysterol formation in the lung impacts those adverse events.

1.6.1 Airway Lining Fluid as a Target for Ozonization

The airway surface liquid (ASL) and airway epithelial cell membranes are rich in cholesterol and other lipid components, which can be directly targeted for oxidation by O_3 (96,97). As Figure 1.7 depicts, the ASL is mostly comprised of phospholipids with smaller contributions of surfactant proteins and cholesterol. Previous studies have demonstrated that the formation of lipid ozonization products (LOPs) can mimic many of the adverse health effects observed after exposure to O_3, however, the involvement of cholesterol ozonization products has not been fully examined (98-100). Cholesterol makes up approximately 10% of the mass of human pulmonary surfactant but is 80-90% of the neutral lipid fraction (64,101). Ozonolysis of cholesterol differs from other phospoholipids located in the ASL, as O_3-derived oxysterols are formed into aldehyde and ketone electrophiles and LOPs undergo free radical chain initiation and lipid peroxidation chain propagation (98). Previous O_3 exposure studies utilizing human pulmonary surfactant and bronchoalveolar lavage fluid exposed to O_3, as well as tissue from exposed mice and rats, show the cholesterol 5,6-double bond and concomitant vinylic methylene group moieties of cholesterol are particularly susceptible to oxidation, resulting in the formation of oxysterols (66,67,102-104). Ozonization of the ASL creates unique and highly reactive oxysterol species such as SecoA and SecoB which have heightened biochemical potential to interact with components of the healthy cell, including adduction to cellular proteins.

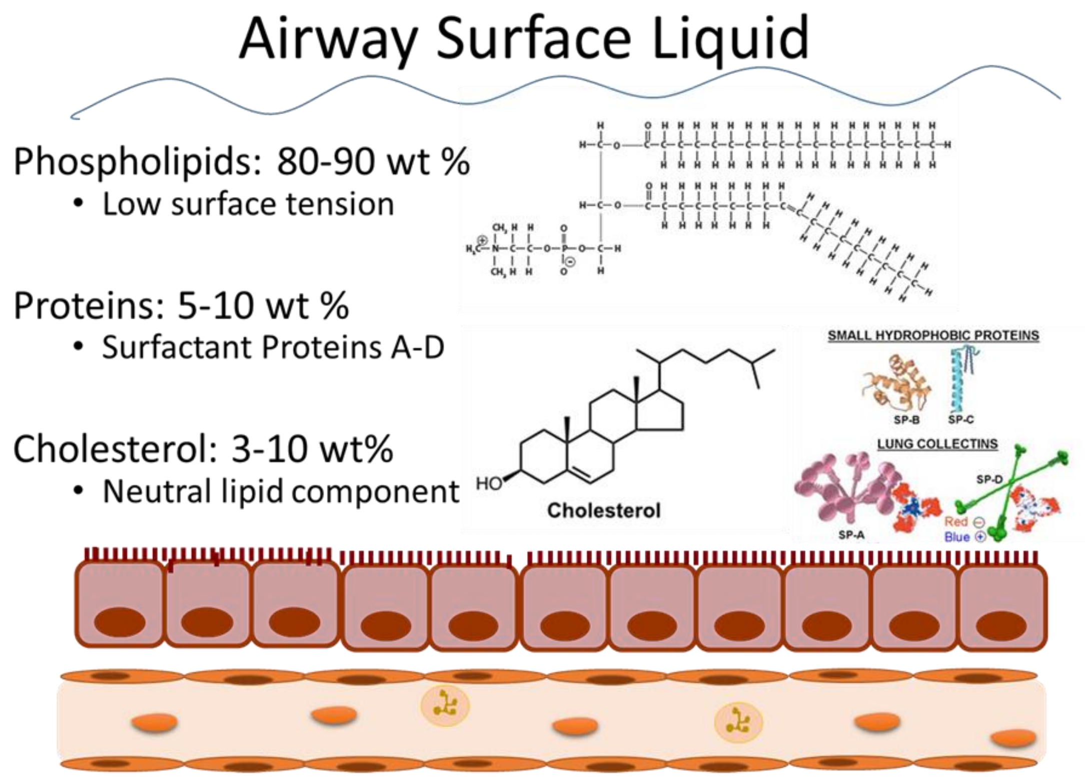

Airway Surface Liquid
Phospholipids: 80-90 wt %
• Low surface tension
Proteins: 5-10 wt %
• Surfactant Proteins A-D
Cholesterol: 3-10 wt%
• Neutral lipid component
HO
Cholesterol
SMALL HYDROPHOBIC PROTEINS
SP-B
SP-C
LUNG COLLECTINS
SP-D
SP-A
Red ⊖
Blue ⊕

Figure 1.7: Constituents of the Airway Surface Liquid

1.6.2 Oxysterol Potential for Protein Adduction

Numerous *in vitro* and *in vivo* studies have demonstrated that exposure to O_3 causes rapid activation of the NF-κB signaling pathway, generation of pro-inflammatory mediators, and infiltration of neutrophils into the airways. Yet, mechanisms linking the known chemical reactivity of O_3 and its reaction products with biological effects continue to present a knowledge gap. A series of studies suggest that ozonized phospholipids could recapitulate O_3-induced activation of NF-κB and pro-inflammatory mediator production (99,100). Similarly, 4-hydroxynonenal (4-HNE), formed during phospholipid peroxidation, causes covalent modification of proteins and also mimics O_3-induced biological effects (105,106). Further, our collaborator Dr. Ned Porter and his group has discovered that O_3-derived electrophilic oxysterols react with nucleophilic centers of proteins (largely lysine residues), forming oxysterol-protein adducts, which could affect the function of these proteins (93), thus providing a new paradigm linking O_3-induced chemical modification of lipids with changes in protein function. While a number of studies, including our own, demonstrate modification of specific proteins with cholesterol ozonolysis products (68,93,107,108), much remains to be learned about the identity of the reactive species, the range of potential proteins modified by these oxysterols in the lung, and the associated biological consequences.

Studies in various cell types have shown that O_3-derived oxysterol species readily react with the nucleophilic lysine, cysteine, and histidine residues of proteins to form covalent linkages (109-112). Specifically, the aldehyde moiety of secosterols has an affinity for adduction to nucleophilic lysine residues in a number of proteins (113). The covalent adduction is characterized by a secure N=C linkage. This strong covalent modification makes the protein more hydrophobic and is capable of inhibiting a protein's localization and/or altering its normal function (114).

1.6.3 Drug-x-Environment Interaction: Impact on Oxysterol Synthesis

Previous studies have shown drug x pollutant interaction involving cholesterol modification and air pollutants. Statins, a known cholesterol modulator drug, have been shown to protect against particulate matter air pollution induced inflammation; however, the mechanism of interaction is not fully understood (71,115,116). Additionally, some data exist indicating that Acetaminophen treatment has the potential to interact with O_3 exposure, exacerbating drug induced liver injury in mice (117). Both of these examples highlight the impact pollutants and pharmaceuticals alone can have on oxysterol formation and associated cellular functions. Thus, there is potential that a synergistic effect between O_3 and cholesterol modulating drugs could have a heightened impact on sterol formation and inflammation. Because of the potential link between lipid metabolism and pollutant-induced inflammation, foods or drugs known to reduce systemic cholesterol levels have been explored for attenuating pollutant or O_3-induced adverse health effects (118-121). Despite these studies, little data has been recorded examining drug x pollutant interaction and respiratory inflammation. Since circulating plasma lipoproteins present the major source of lipids in the lung, dietary contributions and overall systemic lipid metabolism status are likely the predominant factors determining lung cholesterol levels (122). Our primary research discussed in chapter 4 goes further by determining if commonly prescribed drugs that modify sterol levels like APZ can potentiate the already well described oxysterol formation and inflammatory response caused by exposure to O_3.

1.6.4 Specific Aims of this Study and Overall Impact

O_3 is a global air pollutant that causes adverse health outcomes, such as decreased lung function, increased airway inflammation, and exacerbating pre-existing diseases such as asthma. Although the inflammatory response and adverse health effects of O_3 exposure have been characterized, the underlying biochemical and cellular mechanisms by which O_3 mediates adverse health effects and inflammation remain poorly understood. I hypothesize that one mechanism by which O_3 may induce adverse health effects is via oxysterol formation in the airway. O_3-derived oxysterols are electrophilic compounds capable of reacting with nucleophilic residues and forming lipid-protein adducts on a wide variety of protein species. Further, studying mechanisms associated with O_3-derived oxysterol induced health effects can provide a basis to understand susceptibility factors and potential therapeutic interventions. Thus, my studies examining how O_3-derivied oxysterols affect inflammatory pathways may uncover mechanisms by which these drugs modify respiratory responses to O_3.

Therefore, my general **hypothesis is that O_3-derived oxysterols adduct to cellular proteins, activating pro inflammatory signaling cascades and that these responses can be modified by cholesterol-altering drugs, such as small molecule antipsychotic drugs.** The following specific aims are intended to test my central hypothesis: (SA1) Identify oxysterol species formed following O_3 exposure and determine their potential to adduct to protein targets. My preliminary results describe a variety of oxidized cholesterol species in the bronchoalveolar lavage fluid from human volunteers exposed to O_3 *in vivo* and epithelial cells exposed to O_3 *in vitro*. My data also demonstrate lipid-protein adduction between specific oxysterols and cellular proteins. I expand these initial observations and assess the “adductome” using “click” cycloaddition followed by proteomics analysis, addressed in chapter 3. (SA2) Characterize the mechanisms by which O_3-derived oxysterols impact cellular response. To determine if O_3-

derived oxysterols are capable of inducing inflammation in airway epithelial cells by adducting to specific signaling molecules, we examine oxysterol-protein adduction of specific NF-κB inflammation and inflammasome activation pathway proteins, addressed in chapter 3. (SA3) Determine if cholesterol-modifying drugs, such as small molecule antidepressants, modulate O_3-induced inflammation. For example, small molecule antidepressant drugs (e.g. aripiprazole, trazodone) are known to increase overall cholesterol load, yet whether these drugs sensitize individuals to O_3-induced adverse health effects is unknown. We investigate how these drugs modify O_3-induced oxysterol formation and enhanced inflammatory signaling in airway epithelial cells, informing future in vivo and clinical studies, addressed in chapter 4.

The guiding hypothesis of this research is that exposure of the airways to O_3 generates oxysterols at or near the epithelial surface that adduct to cellular proteins to modify cellular signaling and increase airway inflammation. Using the findings developed in this study, we uncover novel interactions between oxidized lipids and cell function in settings of oxidant exposures with relevance beyond O_3. Additionally, our findings foster a better understanding of the potential for pharmaceutical drugs to interact with environmental oxidant exposures. Observations gained from my studies will provide the basis for subsequent human *in vivo* studies determining the interaction between cholesterol-modifying drugs and pollutant-induced lung inflammation, thus uncovering potential drug-pollutant interactions, which can modulate the risk of adverse health effects.

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CHAPTER 2. – OZONE-DERIVED OXYSTEROLS AFFECT LIVER X RECEPTOR (LXR) SIGNALING: A POTENTIAL ROLE FOR LIPID-PROTEIN ADDUCTS.

2.1 Overview

When inhaled, ozone (O_3) interacts with cholesterols of airway epithelial cell membranes or the lung lining fluid, generating chemically reactive oxysterols. The mechanism by which O_3-derived oxysterols affect molecular function is unknown. Our data show that *in vitro* exposure of human bronchial epithelial cells to O_3 results in the formation of oxysterols, epoxycholesterol-α and β (α-EpCh, β-EpCh) and Secosterol A and B (Seco A, SecoB), in cell lysates and apical washes. Similarly, bronchoalveolar lavage fluid obtained from human volunteers exposed to O_3 contained elevated levels of these oxysterol species. As expected, O_3-derived oxysterols have a pro-inflammatory effect and increase NF-κB activity. Interestingly, expression of the cholesterol efflux pump ATP-binding cassette transporter 1 (ABCA1), which is regulated by activation of the liver X receptor (LXR), was suppressed in epithelial cells exposed to O_3. Additionally, exposure of LXR knockout mice to O_3 enhanced pro-inflammatory cytokine production in the lung, suggesting LXR inhibits O_3-induced inflammation. Using alkynyl surrogates of O_3-derived oxysterols, our data demonstrate adduction of LXR with Seco A. Similarly, supplementation of epithelial cells with alkynyl-tagged cholesterol followed by O_3 exposure causes observable lipid-LXR adduct formation. Experiments using Seco A and the LXR agonist T0901317 (T09) showed reduced expression of ABCA1 as compared to stimulation with T09 alone, indicating that Seco A-LXR protein adduct formation inhibits LXR activation by traditional agonists. Overall, these data demonstrate that O_3-derived oxysterols have pro-inflammatory functions and form lipid-protein adducts with LXR, thus leading to suppressed cholesterol regulatory gene expression and providing a biochemical mechanism mediating O_3-derived formation of oxidized lipids in the airways and subsequent adverse health effects.

2.2 Introduction

Currently, the oxidant gas ozone (O_3) is the most widespread air pollutant found in the U.S. and contributes to a growing variety of adverse health outcomes (1). O_3 exposure causes decreased lung function and increased airway inflammation, which exacerbates pre-existing diseases such as asthma and may contribute to certain cardiovascular diseases, all potentially increasing the risk of premature death (2). Although the inflammatory response and adverse health effects of O_3 exposure have been documented for decades, the biochemical and cellular mechanisms by which O_3 mediates adverse health effects remain poorly understood. As a very potent oxidant gas, O_3 reacts readily with the surface components of the airway and causes cellular modification through reactions with the airway lining fluid and epithelial cellular membranes (2-4). The lung lining fluid and epithelial cell membranes are rich in cholesterol and other lipids, which can be directly oxidized by O_3 (5, 6). Previous studies have determined the impact ozonization of lipids, particularly poly unsaturated fatty acids (PUFA), may have on O_3 associated toxic effects. These studies demonstrated that ozonization of PUFAs and the formation of lipid ozonization products (LOPs) can mimic many of the adverse health effects observed after exposure to O_3 (5, 7-9). However, the involvement of cholesterol ozonization products requires further study. The cholesterol 5,6-double bond and concomitant vinylic methylene group moieties are particularly susceptible to oxidation, resulting in the formation of oxysterols (5, 10-12).

Many oxysterols are electrophiles capable of reacting with nucleophilic residues on proteins and other biological macromolecules. They are formed endogenously through enzymatic and non-enzymatic reactions. P450 cytochrome enzymes metabolize endogenous cholesterol into oxysterol species such as 27-hydroxycholesterol, an essential oxysterol for cellular cholesterol homeostasis (13). Studies show that endogenous oxysterols can act as both agonists and antagonists for transcription factors such as the liver X receptor (LXR). Within the LXR protein, a ligand binding domain has been characterized and shown to bind to synthesized oxysterol species *in vitro*, which is the same region that is the target for various post translational modifications such as acetylation and SUMOylation (14). LXR regulates the synthesis of cholesterol transport proteins, such as ATP-binding cassette transporter A1 (ABCA1). ABCA1 transports cholesterol across the cell membrane and shuttles it onto apolipoproteins, resulting in the formation of high-density lipoproteins (HDL). Systemically, failure to produce adequate ABCA1 reduces the ability to generate and transport HDL and is associated with early onset cardiovascular disease, supporting the protective role of ABCA1 in cholesterol metabolism and atherosclerosis (15). In addition to lipid metabolism, both LXR and ABCA1 have been implicated in anti-inflammatory functions (16-19). Moreover, exogenously derived oxysterols can cause adverse cellular effects and have been shown to modulate host immune cell and inflammatory responses (20-22). Oxidation of endogenous cholesterols by various reactive oxygen species (ROS) results in the formation of oxysterols associated with various pathologic processes driven through interaction with the nucleophilic domains of key proteins (23). Elevated levels of specific oxysterol species have been linked to a variety of adverse biological activities including cytotoxicity, increased inflammation, and amplified infection (24-26).

Rodent exposure studies and *in vitro* studies using human pulmonary surfactant have demonstrated the formation of O_3-derived oxidized cholesterol products in various chemical compositions (5, 6, 10, 27, 28). Figure 2.1 depicts the primary reactive O_3-derived oxysterol products Secosterol A (Seco A) along with its aldol condensation product Secosterol B (Seco B), and epoxycholesterols α and β (α-EpCh, β-EpCh) (11, 29). Some of these O_3-derived oxysterols have been found in atherosclerotic tissue and cause foam cell formation, a hallmark of plaque buildup (26). Despite increased interest in the role of oxysterols in human health, especially cardiovascular diseases, information about O_3-derived oxysterols in the airway, their cellular targets, and their biochemical interactions are very limited.

We hypothesize that exposure to O_3 generates lipid-derived electrophiles in the human airway, including reactive species of oxysterols, at or near the epithelial surface that can adduct to cellular proteins, thus ultimately affecting cellular function and inflammatory response. Our study is designed to uncover electrophilic interactions between O_3-derived oxysterols and proteins as a new mechanistic paradigm mediating adverse health effects induced upon inhalation of O_3.

2.3 Materials and Methods

Reagents - Alkynyl-probes were synthesized following the procedures published elsewhere (29, 32). DTT and iodoacetamide were purchased from Sigma Aldrich (St Louise, MO). Streptavidin beads were purchased from GE Healthcare (Piscataway, NJ). The following reagents were purchased from their respective companies: 10% NuPage Novex Bis-Tris® precast mini gel (Invitrogen, Grand Island, NY), PVDF membrane and Simply Blue (Bio Rad, Hercules, CA), IRDye® 800CW Streptavidin (925-32230, Li-Cor, Lincoln, NE), Blocking buffer

(Rockland, Gillbertsville, PA or Odyssey Blocking buffer, Li-Cor, NE). Sequencing grade Trypsin (Promega V5111, Madison, WI). Antibodies of HSP90, LXR-β, PPAR-γ, and Actin were from Santa Cruz (Dallas, Texas) and, LXR-α was purchased from Abcam® (Cambridge, MA).

Cell Culture - 16HBE14o (16HBE) cells, a SV-40 transformed human bronchial epithelial cell line were a gift from Dr. D. C. Gruenert (University of California San Francisco, San Francisco, CA). 16HBE cells were plated on fibronectin-coated (LHC Basal Medium [Life Technologies, Carlsbad, CA], 0.01% BSA [Sigma, St. Louis, MO], 1% Vitricol [Advanced Bio Matrix, San Diego, CA], and 1% human fibronectin [BD Biosciences, San Jose, CA]) 0.4µm Transwell® plates(Costar, Corning, NY), and grown submerged in minimal essential media (Gibco) with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine (Life Technologies) until confluent for 6 days, and 1 day at air-liquid interface (ALI) before use. Primary human bronchial epithelial cells (HBEC) were obtained from healthy donors in collaboration with the Environmental Protection Agency (EPA) using a protocol approved by the University of North Carolina at Chapel Hill Institutional Review Board (Chapel Hill, NC), as described previously (66) The HBECs were cultured in Pneumocult medium for 21 days to differentiate according to previously described methods (67, 68).

In Vitro O$_3$ Exposure - Cultures at ALI were exposed to filtered air or 0.4ppm O$_3$ for 4 hours in exposure chambers operated by the U.S. EPA, as previously described (69). The dose was selected for maximal innate immune response to O$_3$ with minimal cytotoxicity and has been used previously by our group (68). At 1 or 24 h after exposure, apical sides of all cultures were washed with 100 □L Hanks' balanced salt solution (HBSS) (Life Technologies) and saved for LC-MS analysis. The remaining cells were collected in 200 □L PBS, centrifuged at 500 g for 5 min, and were stored at -80 °C until LC-MS analysis.

In Vivo Exposure of Healthy Volunteers to O_3 - Written informed consent was provided by each participant. Healthy volunteers were randomly exposed to air and (in a separate exposure) 0.3 ppm O_3 for 2 h with exercise, with a minimum 2-week separation between exposures in collaboration with the U.S. EPA using a protocol approved by the University of North Carolina Chapel Hill Institutional Review Board, as described previously(70). Bronchoscopy was performed 1 and 24 h after exposure. Cell-free bronchoalveolar lavage fluid (BALF) was stored at -80°C pending analysis (30).

Real-time qPCR - Total RNA was isolated from 16HBEs and HBECs with the use of the Pure Link RNA Mini Kit (Life Technologies, Carlsbad, CA). First strand cDNA preparation and real-time quantitative PCR (qPCR) were performed as previously described (71, 72). Primers and probes for *ABCA1*, *ABCG1*, *FASN*, *SREBP1*, and *β-actin* were commercially available (Applied Biosystems, Foster City, CA). Human *IL8*: 5’-FAM-CCTTGGCAAAACTGCACCTTCAC-TAMRA-3’ (probe), 5’-TTGGCAGCCTTCCTGATTTC-3’ (sense), and 5’-TATGCACTGACATCTAAGTTCTTTAGCA-3’ (antisense) and *IL6*: 5’-FAM-CCAGCATCAGTCCCAAGAAGGCAACT-TAMRA-3’ (probe), 5’-TATGAAGTTCCTCTCTGCAAGAGA-3’ (sense), and 5’-TAGGGAAGGCCGTGGTT-3’ (antisense) were prepared in-house. Differences in expression were determined with the ΔΔCt method and *β-actin* for normalization. Briefly, threshold cycle (Ct) value for the housekeeping gene (*β-actin*) was subtracted from the Ct value for the gene of interest to determine the □Ct value. For each pairwise set of samples to be compared, the difference in ΔCt values between the two samples were calculated for the genes of interest to determine the ΔCt value. The fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$.

Sterol profiling using PTAD derivatization and LC-SRM Analysis. Cells were scraped into 400 μL cold PBS. 200 of the 400 μL were taken and internal standards added (13 ng for d_7-7-DHC, 97 ng for $^{13}C_3$-Desmosterol and 99 ng for $^{13}C_3$-Lanosterol, and 342 ng for d_7-Cholesterol/sample), 10 μL butylated hydroxytoluene (BHT)/triphenylphosphine (TPP) solution (2.5 mg TPP and 1 mg BHT in 1 mL MeOH), 400 μL 1% NaCl, and 500 μL Folch solution (2:1=$CHCl_3$:MeOH). Cells and standards were vortexed vigorously and centrifuged at 3099 x g for 5 min. $CHCl_3$ layer was removed and added to PTAD-predeposited tube (200 μg/tube). The sample tubes were vortexed and analyzed by LC-MS using the following conditions: 10 μL was injected onto the column (Acquity UPLC BEH C18, 1.7 μm, 2.1 x 50 mm) with 100% MeOH (0.1% acetic acid) mobile phase for 1 min runtime at a flow rate of 300 μL/min. The monitored transitions included: 7-DHC m/z 560→365, d_7-7-DHC m/z 567→372, desmosterol m/z 592→365, $^{13}C_3$-desmosterol m/z 595→368, lanosterol m/z 634→602, $^{13}C_3$-lanosterol m/z 637→605 with retention times of 0.8, 0.5, and 0.6 min, respectively.

Oxysterol Extraction from 16HBE or HBEC cells. - To the cell pellets, 10 μL alkynyl-Cholesterol (*a*-Chol) (25ng/μL), 500 μL NaCl (0.9%), 10 μL of TPP and BHT (25 mg TPP and 10 mg BHT in 10 mL MeOH), 1 mL of Folch solution (2:1=$CHCl_3$:MeOH) were added. The mixture was mixed vigorously by vortex for 2 min and separated by centrifugation with 2300 x g for 3 min. The collected organic layer (bottom layer) was evaporated to dryness in a SpeedVac**™** concentrator and resuspended in 100 μL MeOH for LC-MS analysis.

Oxysterol Extraction from Cell-free Bronchoalveolar Lavage Fluid (BALF). - To 1 mL BALF, 10 μL of alkynyl-Seco B (*a*-Seco B) (50 ng/μL), 1mL NaCl (0.9%), 10 μL of TPP and BHT (25 mg TPP and 10 mg BHT in 10 mL MeOH), 2 mL of MeOH, and 3 mL of iso-octane were added. The mixture was vortexed vigorously for 2 min and separated by centrifugation. The collected organic layer was evaporated to dryness in a SpeedVac**™** concentrator and resuspended in 100 µL MeOH for LC-MS analysis.

LC-MS Analysis - The resuspended samples were chromatographed by RP-HPLC using a UPLC BEH C18 column (1.7 µm, 2.1 mm × 100 mm) in Waters Acquity UPLC system equipped with an autosampler (Waters, Milford, MA) and either ESI or APCI in positive ion mode. For ESI, the oxysterols were separated by 95% solvent B in an isocratic method with a flow rate of 200 □L/min, and the mobile phase solvents consisted of 2 mM NH_4OAc (solvent A) in water and 2 mM NH_4OAc in MeOH (solvent B). The injection volume was 10 µL using a partial loop with needle overfill mode. MS detections were done using a TSQ Quantum Ultra tandem mass spectrometer (ThermoFisher, Waltham, MA), and data was acquired and analyzed using a Thermo Xcalibur**™** 2.2 software package. The cholesterol and oxysterols form $[M+NH_4]^+$ ions in positive ion mode (10). The transitions monitored were *m/z* 436→383 for Seco A/B, *m/z* 418→365 for *a*-Seco A/B*, m/z* 404→369 for cholesterol (Chol), *m/z* 386→351 for *a*-Chol, *m/z* 420→385 for EpCh, *m/z* 402→367 for *a*-EpCh. For APCI, 95% MeOH in H_2O containing 0.01% acetic acid was used as a mobile phase. The cholesterol and oxysterols form $[M+H]^+$ ions in positive ion mode. The transitions were *m/z* 369→369 for Chol, *m/z* 383→383 for Seco A/B, *m/z* 351→351 for *a*-Chol, *m/z* 385→385 for EpCh. The transitions of cholesterol and cholesterol esters were *m/z* 365 → 365 monitored by HPLC-MS following the method described elsewhere (73). The amount of the cholesterol esters were found to be less than 2% of free cholesterol in the cells studied.

Cytokine Analysis: Concentration of IL-6 and IL-8 in the apical wash of 16HBE cells exposed to O_3 was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions (BD Biosciences, San Jose, CA).

Western Blotting: Cell lysates from 16HBE cells exposed to air/O_3 or O_3 derived oxysterols were separated by 10% SDS-PAGE and transferred to nitrocellulose. Proteins were detected using specific antibodies (Santa Cruz, Dallas TX) to ABCA1 (1:500) or β-actin (1-2000), which served s a loading control. Antigen-antibody complexes were incubated with horseradish peroxidase-conjugated secondary antibody and were detected using chemiluminescence.

Murine Whole Body O_3 Exposure, BAL, Cytokine and RNA Analysis: C57BL/6J (WT) female mice, 8-12 weeks old and weighing 15-20g, were purchased from Jackson Laboratories (Bar Harbor, ME). LXR$\alpha^{-/-}$ female mice, 8-12 weeks old and weighing 15-20g, originally a kind gift from Dr. David Mangelsdorf, were bred in-house and backcrossed >8 generations onto a C57BL/6 background before use. All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences. Mice were placed in stainless steel wire exposure chambers inside a plexiglass chamber and exposed to filtered air or O_3 for 3 h at a dose of 2 ppm. O_3 was generated by directing 100% oxygen through an ultraviolet light generator and mixed with a humidified air supply to the chamber. Temperature and humidity of chamber air are monitored continuously, as was the O_3 concentration with a Teledyne T400 ultraviolet light photometer. BALF was collected immediately following sacrifice and cell counts performed as described previously (74). Protein analysis was performed using BCA Protein Assay (Pierce, Rockfort, IL.) Cytokine analysis was performed using a Bioplex assay for IL-6 on the BALF (Bio-Rad, Hercules, CA). After necropsy, lung tissue samples were snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. RNA was extracted and cDNA transcribed

(Applied Biosystems, Foster City, CA). 50ng of cDNA was used for qPCR for IL-6 (Mm00446190_m1; Applied Biosystems) and endogenous 18S (4319413) (Applied Biosystems). Ct values were determined using ABI 7500 Real Time PCR System with SDS software 1.3.1. Change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to 18S expression and expressed as fold-change compared to the control group.

Cell Culture and Whole Cell Labeling with a-Seco A in 16HBE - 16HBE cells were plated 2 x 10^6 in 10 cm plates using the conditions described above, then allowed to settle and grow for 24 h. The cells were then incubated in the presence of *a*-Seco A (20 µM) in reduced FBS (2%) MEM medium for 4 h. Cells were harvested in lysis buffer (50 mM HEPES, 150 mM NaCl, 0.1% TritonX100, pH=7.0) containing protease inhibitors (Sigma P8340) on ice. The lysate was cleared by centrifugation at 10,000 x g for 10 min @ 4 °C to remove cellular debris. The total protein concentration was determined using standard BCA assay (Pierce, ThermoFisher) for further click reaction.

Cell Culture and Whole Cell Labeling with a-Cholesterol Followed by Ozone Exposure in Human Bronchial Epithelial Cells (16HBE) - 16HBE cells were grown in 24 mm transwells submerged in MEM with reduced FBS containing 20µM *a*-Chol for 6 days. The apical media was removed 24 h before exposure. Next, the plate was exposed to filtered air or O_3 (0.4 ppm) for 4 h and allowed to continue for an additional 1 h incubation without O_3 (1 h post incubation). Cells were harvested in 300 µL lysis buffer/well on ice and all wells were then combined for each condition. The lysate was cleared by centrifugation at 10,000 x g for 10 min @ 4 °C to remove cellular debris. The total protein concentration was determined using standard BCA assay (Pierce, ThermoFisher) for further click reaction.

Click Biotinylation of a-Seco A/a-oxysterols Adducted Proteins in 16HBE and

Streptavidin Affinity Purification - 1mL of cell lysates (2 mg/mL) were reduced with sodium borohydride, 5 mM final concentration, for 1 h on ice to stabilize the adducts. Excess sodium borohydride was deactivated by acidification of the mixture by adding 1M HCl. Subsequently, all click reagents were added to the reduced cell lysates, including the photo-cleavable azido-biotin (39) (0.2 mM final concentration, tris(3-hydroxypropyltriazoylmethyl)amine (THPTA) (75) (0.2 mM), $CuSO_4$ (1 mM), and sodium ascorbate (1 mM), and the reaction mixture was vortexed and allowed to react at room temperature for 1 h. 50 µL of the reaction mixture was saved for Streptavidin visualization. The rest was precipitated using cold methanol (3/1 = $MeOH/H_2O$, v/v) to remove excess biotin linker. The precipitated protein pellets were reconstituted in 1 mL of 0.1% SDS in PBS including 200 µL of streptavidin (GE Health Bioscience) slurry. The slurry was rotated in the dark for 2h at room temperature to capture the adducted proteins. After 2 h, the tube was spun at 95 x g for 1 min and the supernatant removed (flow-through). The beads were then washed with 1 mL of 1% SDS (x2), 4M urea (x2), 1M NaCl (x2), and 25 mM ammonium bicarbonate (AMBIC, x2) respectively. The slurry was transferred to a 0.2 µm cellulose acetate spin filter (Costar) in 500 µL of 25 mM AMBIC and spun at 95 x g for 1 min. The slurry was re-suspended with AMBIC and photolyzed under hand-held UV light (365 nm) for 2 h with gentle stirring at room temperature. The spin filter was spun at 95 x g for 1 min to recover photo-released proteins. The beads were washed twice with 500 µL of 25 mM AMBIC and the combined filtrates were evaporated to dryness in a SpeedVac™ concentrator.

Visualization of Biotinylated Proteins Adducted with a-SecoA/a-Oxysterols - The saved 50 µL of click-reaction mixture was mixed with SDS sample loading buffer and resolved using 10% NuPAGE Novex BisTris® gel (Invitrogen, Carlsbad, CA). The proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules CA) and probed with IRDye® 800CW Streptavidin (Li-Cor, Lincoln, NE). The extent of adduction was visualized using Odyssey Infrared Imaging System™.

Immunoblotting Analysis of Photo-released Proteins - The photo-released and dried proteins were reconstituted in 70 µL PBS, 25 □L LDS buffer, and 5 µL of 1M DTT and resolved by 10 % NuPAGE Novex BisTris® gel then transferred to PVDF membrane. The transferred proteins were incubated with antibodies of HSP90 (anti-rabbit, Santa Cruz), LXR-α (anti-rabbit, Abcam®), LXR-β, (anti-rabbit, Santa Cruz) or PPAR-γ (anti-mouse, Santa Cruz) (1:1000) overnight in the cold room at 4°C. Alexa Fluor 680® labeled secondary anti-rabbit or mouse was used to detect target proteins. Immunoreactive proteins were visualized using Odyssey Infrared Imaging System™ (Li-Cor, Lincoln, NE).

Modification of Gene Expression by Sequential Seco A - T09 Challenges - 16HBE cells were grown submerged in MEM medium and repeatedly challenged with 20µM Seco A, 10µM T09 or DMSO in a crossover design. Briefly, cells were challenged with the first stimulus for 2 h, washed with HBSS, and challenged again with either 20µM Seco A, 10µM T09 or DMSO for 2 h. The cells were washed again with HBSS and RNA was collected for analysis as described above.

NF-κB and LXRE Promoter Reporter Activity in 16HBE Cells - 16HBE cells were transduced with NF-κB-luciferase lentiviral vector at a multiplicity of infection of 5 and were cultured for seven days (76). To select for transduced cells, the cells were cultured with hygromycin for nine days to create a stable cell line, which was used for subsequent reporter assays. To assess NF-κB activation, stably transduced 16HBE cells were plated overnight and then treated with α-EpCh, β-EpCh, Seco A, SecoB (all 20 µM) or TNF-α (20 ng/mL, Calbiochem, Billerica, MA) for 4 and 24 h. For the LXR response elements (LXRE) promoter activity measurement, LXRE-luciferase vector (System Biosciences Inc, San Francisco, CA) was transiently transfected into 16HBE cells and allowed to expand for at least 1 day. The cells were then exposed to individual oxysterols or used in the sequential challenge with Seco A and T09 as described above. Cell lysates were harvested and subjected to dual luciferase assay (Promega, Madison, WI). Data were normalized to total protein levels and expressed as relative luciferase units (RLU).

Statistical Analysis – All *in vitro* data were performed in at least 3 separate experiments. Data shown are mean ±SEM and significance indicated as $p<0.05$. See figure legends and for further information on the specific statistical analysis used for each experiment.

2.4 Results

Identification of O_3 induced Oxysterol Formation in vitro and in vivo – Primary differentiated HBEC cells and 16HBE cells were chosen to evaluate the presence of oxysterol species *in vitro* following exposure to O_3. Representative HPLC-MS profiles reflected increased levels of oxysterol species in O_3 exposed 16HBE cells (Fig. 2.2A) compared to filtered air-exposed cells. 16HBE cells exposed to O_3 exhibited elevated concentrations of α-EpCh, β-EpCh, and Seco B in cell lysates (Fig. 2.2B) and in the apical washes (Fig. 2.2C) collected both 1 and 24 h post-exposure compared to the air exposed control. A similar trend was observed in primary HBEC lysates (Fig. 2.2D), and apical washes (Fig. 2.2E) at both 1 and 24 h post exposure compared to air exposed controls. Overall, the cholesterol concentration did not significantly change due to O_3 exposure or sample collection time in either 16HBE (4870±597 ng/sample Air to 5168±138 ng/sample O_3) or primary HBE (50121±1554 ng/sample Air to

5506±772 ng/sample O_3) cells.

To test the formation of O_3-derived oxysterols in humans *in vivo*, we determined the levels of oxysterols identified in BALF obtained from healthy volunteers exposed to filtered air or 0.3ppm O_3 for 2 h. The BALF was collected by bronchoscopy at 1 and 24 h post-exposure and various oxysterol species were quantified by HPLC-MS. Exposure to O_3 significantly elevated the concentrations of β-EpCh (Fig. 2.3B) and Seco A (Fig. 2.3C) compared to individuals exposed to filtered air controls at 1 h post-exposure with *p* values of less than 0.001 and 0.05 respectively. A moderately convincing increase ($p<0.1$) was observed for α-EpCh (Fig. 2.3A) and no difference in Seco B (Fig. 2.3D). Unlike the results observed in the *in vitro* samples, we observed that oxysterol concentrations returned to baseline levels 24 h post-exposure. Again, there was no significant change in overall BALF cholesterol concentration in subjects exposed to air (6639±1556 ng/mL) compared to O_3 (6698±2245 ng/mL).

The Effects of O_3 on Cholesterol Efflux and Cytokine Gene Expression – As expected and previously described by us (30, 31), exposure to O_3 caused an inflammatory response as marked by increased gene expression (Fig. 2.4A/B) and protein concentration (Fig 2.4C/D) of pro-inflammatory cytokines IL6 and IL8. Treatment with synthetic LXR agonist T0901317 (T09) alone did not affect the levels of *IL6* or *IL8* expression (Fig. 2.4A/B). Interestingly, exposure to O_3 in the presence of T09 significantly decreased the expression of cholesterol efflux pump protein *ABCG1* (Fig. 2.4E) and *ABCA1* (Fig. 2.4F), suggesting O_3-induced inhibition of the LXR pathway. Additionally, ABCA1 protein levels were significantly reduced in cells exposed to O_3 compared to air as shown in relative densitometry from 3 separate experiments (Fig. 2.4G) and representative immunoblot (Fig. 2.4H).

LXR-$\alpha^{-/-}$ mice are more susceptible to O_3 induced inflammation. Female LXR$\alpha^{-/-}$ were compared to WT mice. Each were exposed to filtered air or 2ppm O_3 for 3 h. BALF was collected from both conditions and evaluated for concentrations of IL-6. The LXR-$\alpha^{-/-}$ mice showed significantly higher concentrations of IL-6 compared to the WT (Fig. 2.5A). At necropsy the lungs were removed and homogenized for RNA isolation and qPCR analysis. In a similar result, IL-6 mRNA levels were significantly higher in the LXR-$\alpha^{-/-}$ mice exposed to O_3 compared to the WT control mice exposed to O_3 (Fig. 2.5B). As seen in the human BALF samples (Fig. 2.3C/D), exposure to O_3 increases the amount of Seco A (Fig. 2.5C) and Seco B (Fig. 2.5D) in the mouse BALF samples with significantly higher amounts of Seco A present in the LXR-$\alpha^{-/-}$ compared to the WT mice. Interestingly, unlike the human samples, total cholesterol levels decreases in O_3-exposed animals (Fig. 2.5E).

Exposure to a-Seco A Reveals LXR-α Protein Adducts – Based on recent studies demonstrating oxysterol-induced lipid-protein adducts, we hypothesized that O_3 derived oxysterols can adduct to the LXR-α and LXR-β proteins, which contain reactive lysine residues (14, 29). Seco A, a primary ozonide and one of the most reactive O_3-derived oxysterols, reacts with proteins and causes covalent modifications leading to lipid-protein adduct formation (32). 16HBE cells were treated with alkynyl-tagged Seco A (*a*-Seco A) and the adducted proteins were identified by first adding biotin to the alkynyl tag utilizing click cycloaddition (Fig. 2.6A) followed by immunoaffinity purification with streptavidin beads and photo-release (Fig. 2.6B), which frees cellular proteins adducted by *a*-Seco A. This mixture of proteins was subsequently subjected to western blot analyses using antibodies against HSP90, LXR-α, LXR-β, PPAR-γ, and β-actin (Fig. 2.6C). HSP90 was used as a positive control based on previous studies (33). Our data show that treatment of bronchial epithelial cells with *a*-Seco A results in the formation of Seco A-LXR adducts.

Alkynyl Cholesterol Supplemented Cells Exposed to O_3 Reveals the Formation of LXR-□ adducts – 16HBE cells were supplemented with 20□M alkynyl-tagged cholesterol (*a*-Chol) for 6 days. Media containing *a*-Chol changed daily with washes on the apical side 24h prior to exposure (Fig. 2.7B). The 16HBE cells were exposed to filtered air or 0.4ppm O_3 for 4 h and harvested 1 h post-exposure. Our data show that *a*-Chol, a surrogate of endogenous cholesterol, is incorporated into the cells and, in response to O_3, generates a host of alkynyl-oxysterols identical to the oxysterol species generated via oxidation of endogenous cholesterol (Fig. 2.7A). Based on streptavidin analysis, cells exposed to O_3 exhibit a higher overall level of protein adduction with oxidized cholesterol species than cells exposed to air (Fig. 2.7C). Similar to cells treated with *a*-Seco A, HSP90 and LXR-α were found to be targets of alkynyl-tagged oxysterols generated endogenously in cells exposed to O_3 (Fig. 2.7D). The broad band of LXR-antibody reactive proteins shown in Figure 2.7D (Elute+O_3) suggests that a set of O_3-derived adducts are formed under conditions of endogenous oxysterol genesis. This is in contrast to the homogenous protein adduct observed after exposure to a single exogenous electrophile, as shown in Figure 2.6C for the experiment with *a*-Seco A. Figure 2.7E shows the densitometric analysis of O_3-derived adducts of LXR-α from 3 separate experiments.

Oxysterol Exposure Alters ABCA1, FASN, and SREBP1 Gene Transcription – Considering the effects of oxysterols on adduct formation with LXR, we evaluated if exposure to oxysterols can alter expression levels of genes controlling cholesterol biosynthesis, fatty acid synthesis and cholesterol efflux in 16HBE cells. To determine whether oxysterol adduct formation with LXR inhibits subsequent activation of LXR, we sequentially treated 16HBE cells with DMSO, Seco A, or T09 for 2 h, removed the first treatment and followed with a second challenge for 2 h (Fig. 2.8A). Treatment with Seco A followed by T09 significantly suppressed

ABCA1 (Fig. 2.8B), *FASN* (Fig. 2.8C), and *SREBP1* (Fig. 2.8D) gene expression compared to cells exposed to DMSO followed by T09 and T09 followed by T09, suggesting that initial treatment with Seco A reduces the ability of T09 to enhance cholesterol synthesis and efflux gene expression. Challenge with individual oxysterol species yielded no significant change in LXRE activity when compared to the DMSO vehicle control (Fig. 2.8E). However, LXRE activity was significantly reduced in cells sequentially treated with Seco A followed by T09 compared to cells sequentially treated with DMSO followed by T09 or T09 followed by T09 (Fig. 2.8F), again suggesting that initial treatment with Seco A reduces the ability of T09 to activate LXR.

Exposure to Individual Oxysterols Activates the Inflammatory Gene Transcription Pathway - In order to determine whether O_3-derived oxysterols modify expression of inflammatory genes alone and in relation to known agonists, 16HBE cells were sequentially treated with DMSO, Seco A or T09 for 2 h followed by a second challenge for 2 h as described above (Fig. 2.8A). Exposure to Seco A followed by T09 increased the expression of *IL6* (Fig. 2.9A) and *IL8* (Fig. 2.9B) compared to the DMSO treated control. Additionally, individual O_3-derived oxysterol species significantly increased the NF-κB activity compared to the DMSO control (Fig. 2.9C), suggesting that O_3-derived oxysterols enhance pro-inflammatory pathways.

2.5 Discussion

Despite a large body of research on O_3-induced toxicity and adverse health effects, the biochemical and cellular signaling cascades induced by O_3 exposure in the human airways are not fully understood. O_3 is a known oxidant of the macromolecules in the airway lining fluid and the formation of many oxidized lipid products has been previously described along with their biochemical effects. For example, instillation of 5beta,6beta-epoxycholesterol (β-EpCh) and 1-palmitoyl-2-(9'-oxo-nonanoyl)-glycerophosphocholine (PON-GPC), which are both formed during O_3 exposure, causes neutrophilic influx, which in turn was regulated by class A scavenger receptors, known to bind oxidized lipids (3). Ozonization of phospholipids generates products with strong pro-inflammatory effects (8, 9). Additionally, O_3 is known to react with lung surfactant proteins, modifying and inhibiting their normal biological functions (34-36). O_3 induced oxidized cholesterol products, however, have only partially been described in their effects on the biological properties of the human airway. Through this study, our results address the knowledge gap between O_3 induced cholesterol oxidation in the airway and downstream cellular signaling events. Our data demonstrate that O_3-derived oxysterols can form lipid-protein adducts with cellular signaling molecules, revealing a new paradigm that lipid-protein adduct formation provides a central mechanism for O_3-derived oxidized lipids to modify cellular responses.

Lipid protein adduction as a means to alter normal signaling has been shown to occur during exposure to other oxidative stressors, including the free radical induced formation of 4-hydroxynoneal (HNE) (37, 38). While formation of oxysterols following exposure to O_3 has been shown before (28, 35), whether and how these O_3-derived oxysterols can form oxysterol-protein adducts in the context of O_3 exposure in the human airway has not been examined. Assays based on “click” cycloaddition methods make it possible to visualize protein-lipid adducts via immunostaining and determine protein adductions with specific signaling molecules, thus providing novel insight into the mechanisms behind O_3-induced adverse health effects (39). Using this approach, our results demonstrate the formation of oxysterol-protein adducts in airway epithelial cells as a result of exposure to O_3. The synthetic O_3-derived oxysterol Seco A has previously been described as a highly reactive ozonolysis product capable of adducting with the lysine amino acid residues on various proteins (29). Through our study, we determined that O_3-derived oxysterols, including Seco A, can effectively form adducts with proteins LXR-α, LXR-β and PPAR-γ, which all regulate lipid metabolism (40, 41). Additionally, supplementation with alkynyl-tagged cholesterol reveals that in the human airway, exposure to O_3 generates reactive oxysterols capable of forming adducts with the LXR-α and HSP90 proteins. The comparatively diffuse band observed in Figure 2.7D and quantitatively analyzed in Figure 2.7E is most likely indicative of a heterogeneous mixture of O_3-derived oxysterols being formed that can adduct LXR and of a variety of post-translational modification to LXR-α in addition to *a*-Seco A adduction resulting in a mixture of proteins recognizable by the anti- LXR-α antibody. Hence, data presented here reveals a novel biochemical mechanism involving the conversion of cellular cholesterol by O_3 into reactive oxysterol species, a process that results in the adduction of key proteins and modification of cellular signaling.

The formation of adducts between O_3-derived oxysterols and LXR provides a model signaling mechanism, which results in modifications of airway inflammatory and cholesterol homeostasis signaling. Endogenous oxysterols are known to be formed during normal cholesterol regulation and metabolism. These compounds play a role in normal LXR activation, promoting cholesterol homeostasis. Mouse model systems with LXR and cholesterol efflux deficiencies have higher cholesterol accumulation, potentiated atherosclerosis, and are more susceptible to systemic infection (42). These effects occur partially via the endogenous oxysterol-induced activation of LXR and transcriptional activation of genes such as *ABCA1*, which mediate cholesterol shuttling and ultimately packaging into HDL (43, 44). In contrast, our data show that in human bronchial epithelial cells, O_3 exposure decreases the expression of *ABCA1*, suggesting that O_3 or its oxidation products have an inhibitory effect on the transcriptional activation of *ABCA1*. Decreased *ABCA1* expression and subsequent decreased cholesterol efflux may result in accumulation of intracellular cholesterol leading to dyslipidemia and propagation of respiratory, metabolic, and cardiovascular health problems (13, 45). Whether the observations related to O_3-induced suppression of *ABCA1* expression affects systemic cholesterol metabolism and potentially contributes to the enhanced atherosclerosis and cardiovascular events associated with O_3 exposure remains to be established. In addition to cholesterol homeostasis, previous studies have indicated that normal LXR activation is necessary for immune health. LXR activation by synthetic agonists leads to decreased neutrophil recruitment and increased bacterial burden, indicating that LXR activation balance contributes to host immune defense (46). Recent studies also suggest that LXR agonists show therapeutic promise in treating lung disorders such as dyslipidemia and asthma (43, 47, 48). Further, our data show that O_3 exposure of LXRα$^{-/-}$ mice results in enhanced pro-inflammatory response, indicating the importance of normal LXRα activity to regulate O_3 induced inflammation (Fig. 2.5A/B). The enhanced concentration of O_3 derived oxysterols in the BAL sampled from the LXRα$^{-/-}$ mice further link airway cholesterol ozonolysis and LXRα activity. It is interesting that overall cholesterol levels were reduced in these mice exposed to O_3, a change we did not observe in our human subjects BALF. Taken together, LXR dysfunction caused by O_3-derived oxysterols may contribute to enhanced respiratory inflammation and adverse cardiovascular health effects in humans exposed to O_3.

Based on our observation of adduct formation between O_3-derived oxysterols and LXR, we hypothesize that electrophilic O_3-induced oxysterols and their derived lipid protein adducts inhibit cholesterol signaling pathways that are normally activated by endogenous oxysterols. Our data in Figure 2.8E show that O_3-derived oxysterols alone are not themselves potent activators of

LXR , but that treatment with Seco A prior to activation of LXR by the agonist T09 reduces T09-induced LXRE activation as well as expression of *ABCA1, FASN, and SREBP1*. These data suggest that Seco A-induced adduct formation with LXR may prevent its activation with known agonists, such as T09, or endogenous oxysterols, such as 27-hydroxycholesterol (49). Our previous studies have demonstrated that Seco A readily adducts to nucleophilic lysine amino acid residues in human serum albumin and other protein and we hypothesize a similar binding site in LXR (29). LXR contains eight lysines in a ligand binding domain (amino acids 215-434) that are capable of binding both oxysterol and T09 ligands (14). Based on our results, we propose that O_3-derived oxysterols block T09 mediated LXR activation either by non-competitive binding to the same site or by modifying LXR structure to inhibit normal ligand binding. Additional studies are necessary to clarify the nuclelophilic peptide site of O_3-derived oxysterol LXR adduct formation.

In contrast to expression of *ABCA1*, expression of *IL6* and *IL8* as well as activation of NF-κB was enhanced by exposure to Seco A and other O_3-derived oxysterols. O_3 is known to activate NF-κB leading to increased transcription of inflammatory cytokines such as *IL6* and *IL8* (31, 50, 51). Similarly, other lipid ozonization products have been shown to activate *IL6* and *IL8* in human airway epithelial cells (9). Our study shows that O_3 derived oxysterols enhance activation of NF-κB and potentiate the transcription of *IL6* and *IL8*. LXR signaling has been shown previously to have an inhibitory effect on NF-κB and inflammatory signaling in epithelial cells (52, 53), making it a reasonable target for O_3 derived oxysterol-induced modification of inflammatory signaling. All the O_3-derived oxysterol species we tested increased NF-κB activity, revealing that in their presence, airway epithelial cells may experience increased inflammatory signaling leading to adverse health effects. In the experiments using sequential stimulation with

Seco A and T09, stimulation with Seco A alone, regardless of the secondary stimulus, increases *IL6* and *IL8* expression, indicating that O_3-derived oxysterols potentiate the transcription of pro-inflammatory cytokines. In addition to directly activating the NF-κB pathway, we hypothesize that this effect may be linked to inactivation of LXR. Activation of LXR induces a number of anti-inflammatory responses, marked by inhibition of NF-κB activity and decreased production of pro-inflammatory mediators (54). Previous rodent studies show the use of LXR agonists to inhibit NF-κB activation in order to reduce lung injury and inflammation after hemorrhage and resuscitation (55). In this role, LXR is not recruited to LXR response elements in genes related to lipid metabolism, but participates in a transrepression mechanism of NF-κB, resulting in inhibition of inflammatory genes such as *IL-6* (42, 56, 57). LXR activity is controlled by a post-translational SUMOylation modification and interaction with nuclear receptor co-repressors, reducing NF-κB activity. (58-60). The proposed SUMOylation occurs at the lysine residues of LXR ensuring RXR dimerization and nuclear translocation (60). Thus, lipid-adduct formation at or near these lysine residues may prevent SUMOylation of LXR, interaction with co-repressors, and consequently activation of NF-κB. This proposed signaling mechanism provides a link between our observed O_3-derived oxysterol induced changes to LXR signaling and a model for O_3 induced NF-κB activation.

In order to enhance our understanding of O_3 induced cardiovascular events and atherosclerosis, whether the biochemical mechanisms involving O_3-derived oxysterols, adduct formation with LXR, and reduced *ABCA1* expression are reflected in systemic changes needs to be examined. Interestingly, epidemiological studies repeatedly demonstrate the beneficial effects of cholesterol modifying drugs, such as statins, on air pollutant-induced adverse health effects (61-65). Yet, the mechanisms mediating these effects are unknown and have often been ascribed to the anti-inflammatory effects of statins. The findings presented in this study provide an additional explanation for the beneficial effects of statins on the response to inhaled O_3. Therapeutic treatment with lipid-targeting agents such as statins may reduce cholesterol availability in the airways, thus reducing oxysterol formation, and leading to changes in O_3 induced inflammation.

Collectively, our results suggest that reactive oxysterols formed in the airways following O_3 exposure interact with the LXR signaling pathway, alter expression of genes that regulate cholesterol efflux, fatty acid synthesis, and cholesterol regulation, and enhance pro-inflammatory signaling pathways known to be associated with O_3 exposure. Our findings indicate that cholesterol availability is paramount to the formation of O_3-derived oxysterols in the airway and may provide a novel therapeutic target to reduce the adverse effects associated with ambient O_3 exposure. In addition to LXR examined here, other proteins are also likely targets for adduction by O_3-derived oxysterols and warrant future study into their implications in O_3 induced airway inflammation. Together, our data describe a novel mechanistic concept linking O_3 reactions with cholesterol moieties in the human airway and its impact on health outcomes. Our data also highlight the use of samples derived from human *in vivo* O_3 exposure as well as primary HBECs and 16HBE cells to provide clinical and biological relevance for O_3 derived oxysterol and their role in O_3 induced inflammation in the human airway.

2.6 Figures

Figure 2.1: Cholesterol and the major oxysterols formed in the reaction with ozone.

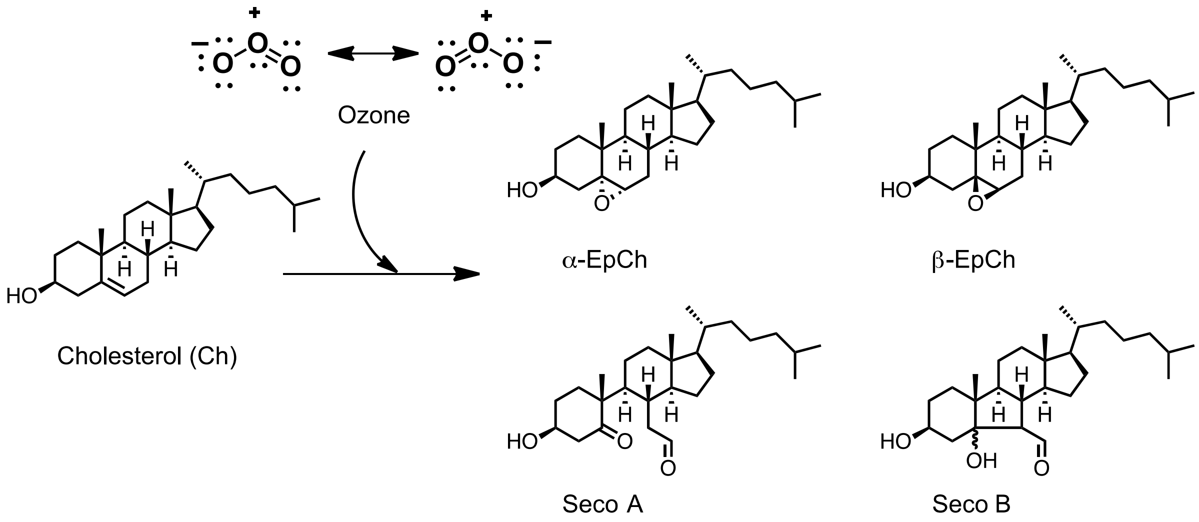

Ozone
Cholesterol (Ch)
α-EpCh
β-EpCh
Seco A
Seco B
HO
OH
O
H

Figure 2.2: Oxysterol concentrations measured in cells and apical washes exposed to filtered air or O_3 and their HPLC-MS profiles. All cells were grown in 24mm Transwell membrane plate until confluency followed by removal of the apical medium and exposure to filtered air or 0.4 ppm O3 for 4 h. Cell lysates and apical washes were collected at 1 and 24 h post-exposure. (A) Representative reverse-phase HPLC-MRM chromatograms of epithelial cells exposed to air or 0.4 ppm ozone. MS for each panel is selected reaction monitoring (SRM) of the m/z indicated in the air exposure. (B) 16HBE cell lysate, (C) 16HBE apical wash, (D) primary HBEC cell lysate, (E) primary HBEC apical wash. Data are presented as mean ± SEM. Statistical analysis was performed with a one-way ANOVA and Fisher's LSD post hoc test comparing observed means against the respective air-exposed control, *p<0.05. n=3.

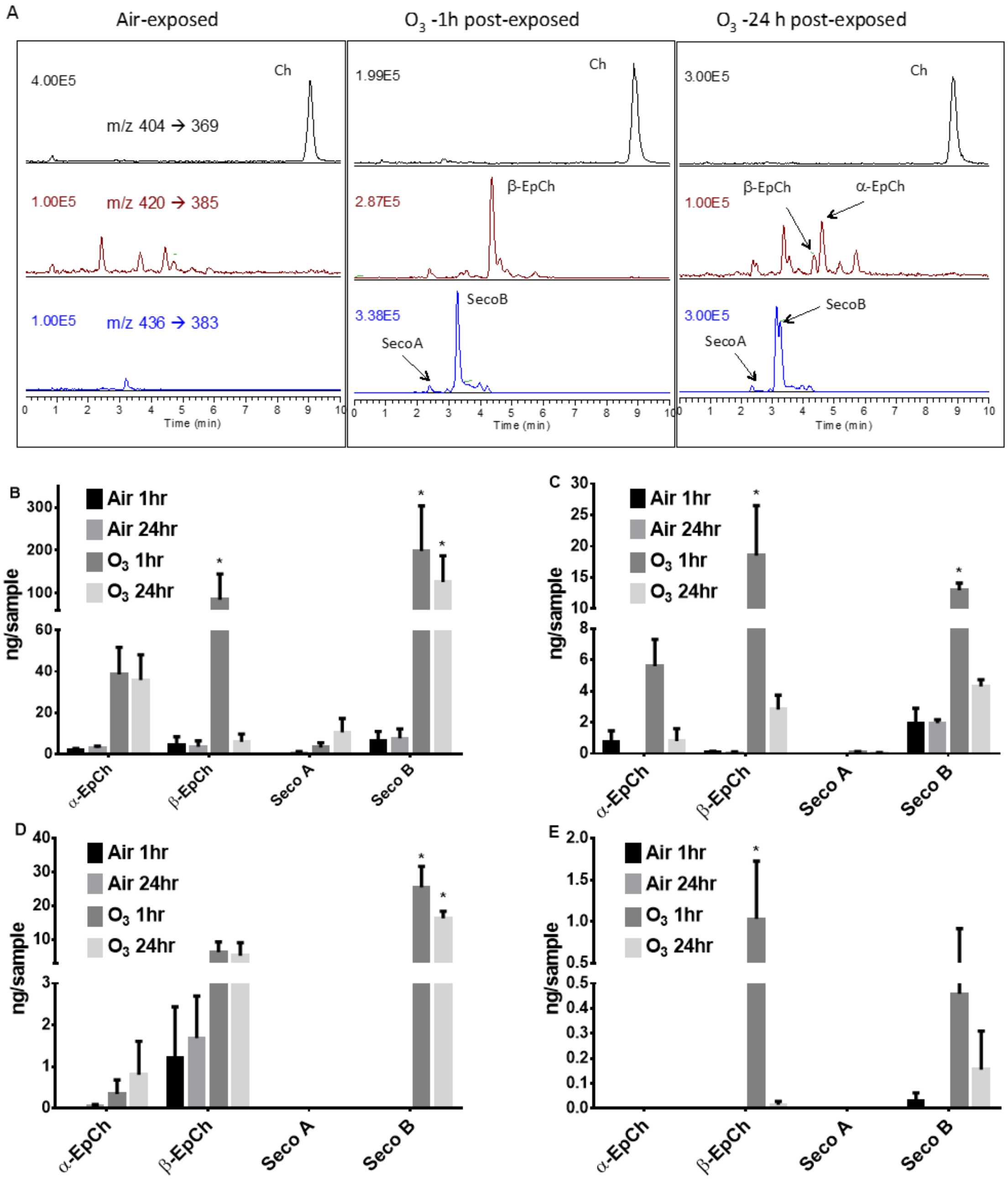


Figure 2.3: Oxysterol concentrations measured in airway BALF Healthy individuals were exposed to either filtered air (control) or 0.3 ppm O3 for 2 h. Cell free BALF was collected by bronchoscopy at 1 and 24 h post-exposure. Oxysterols were quantified by HPLC-MS spectrometry. (A) α-EpoxyCholesterol (α-EpCh), (B) β-EpoxyCholesterol (β-EpCh), (C) Secosterol A (Seco A), (D) Secosterol B (Seco B). Data are presented as mean ± SEM. Statistical analysis was performed with paired student's t-test (two tailed distribution, pairing based on subject). *p<0.05, **p<0.001. n=9-11.

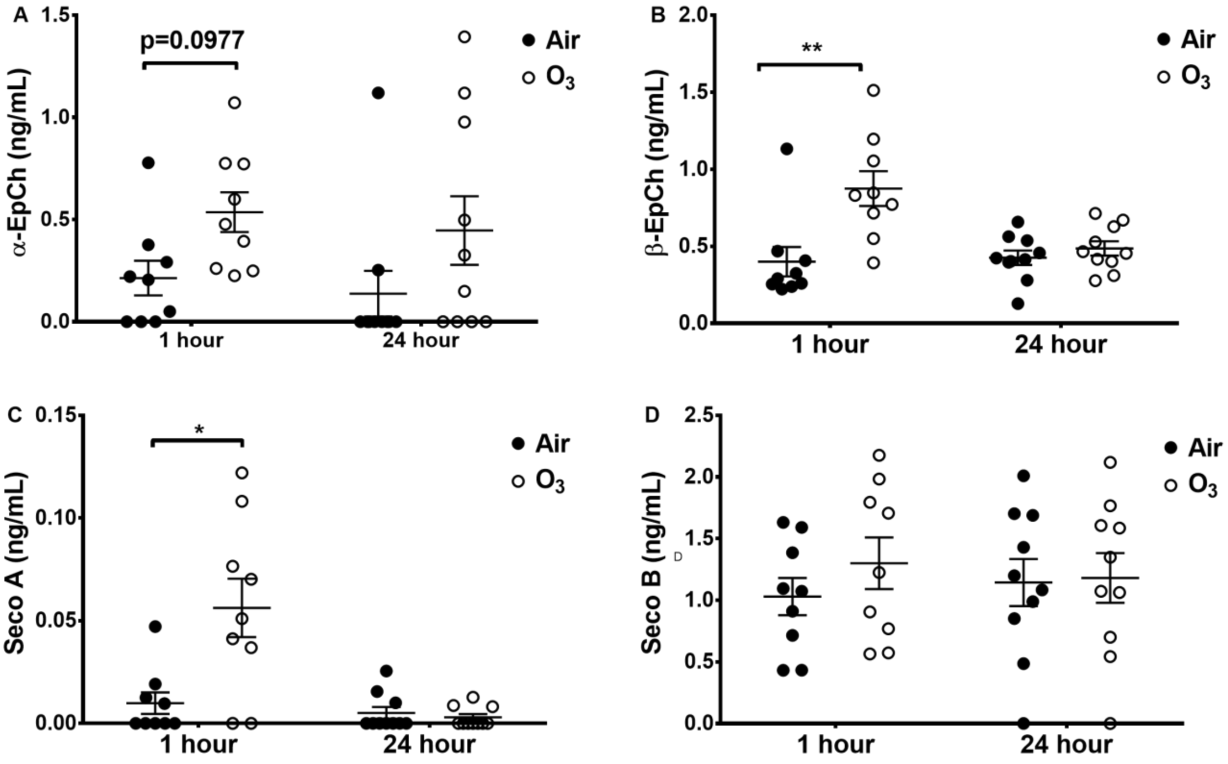


Figure 2.4: Cholesterol efflux pump protein and pro-inflammatory cytokines gene expression levels in 16HBE cells. Cells were exposed to filtered air or 0.4 ppm ozone for 4 h with and without 10μM T09. RNA and apical wash samples were collected 1 h post-exposure and analyzed. Inflammatory gene expression levels (A) IL-6, (B) IL-8, and protein levels (C) IL-6, (D) IL-8; as well as gene expression levels for (E) ABCG1 and (F) ABCA1 were measured. Additionally, ABCA1 protein levels were evaluated by (G) immunoblot and (H) relative densitometry from 3 separate experiments. Data are presented as mean ± SEM. Statistical analysis was performed with a one-way ANOVA and Fisher's LSD post hoc test comparing observed means against the respective treatment/air-exposed control, *p<0.05, or against the DMSO/air exposed vehicle control, #p<0.05, n=3.

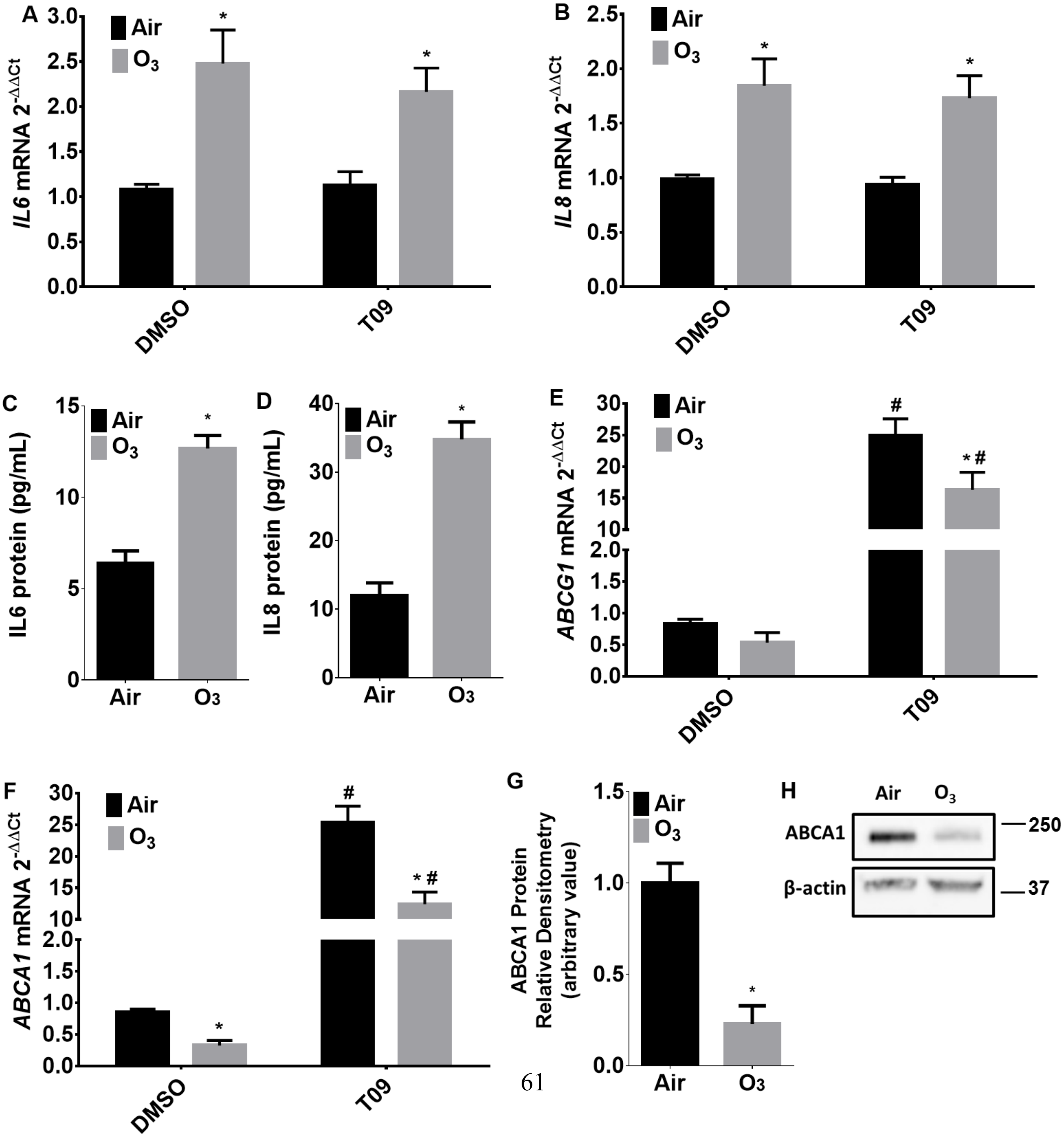


Figure 2.5: IL-6 expression and production in the lung of O_3 exposed LXRα deficient mice. Wild Type (WT) and LXR-α-/- female mice were exposed to filtered air (Air) or 2ppm O_3 for 3 h. Mice were necropsied 6h after start of exposure. (A) Bronchoalveolar lavage was collected and evaluated for IL-6 cytokine concentrations by ELISA. (B) After necropsy lung tissues were collected for RNA isolation and evaluated for gene expression of IL-6 and 18S. Brochoalveolar lavage was also evaluated for (C) total Seco A, (D) total Seco B, and (E) total cholesterol. Data was presented as mean ± SEM. Statistical analysis was performed with Mann-Whitney test comparing LXR-α-/- to WT, *p<0.05, n=5 animals per group.

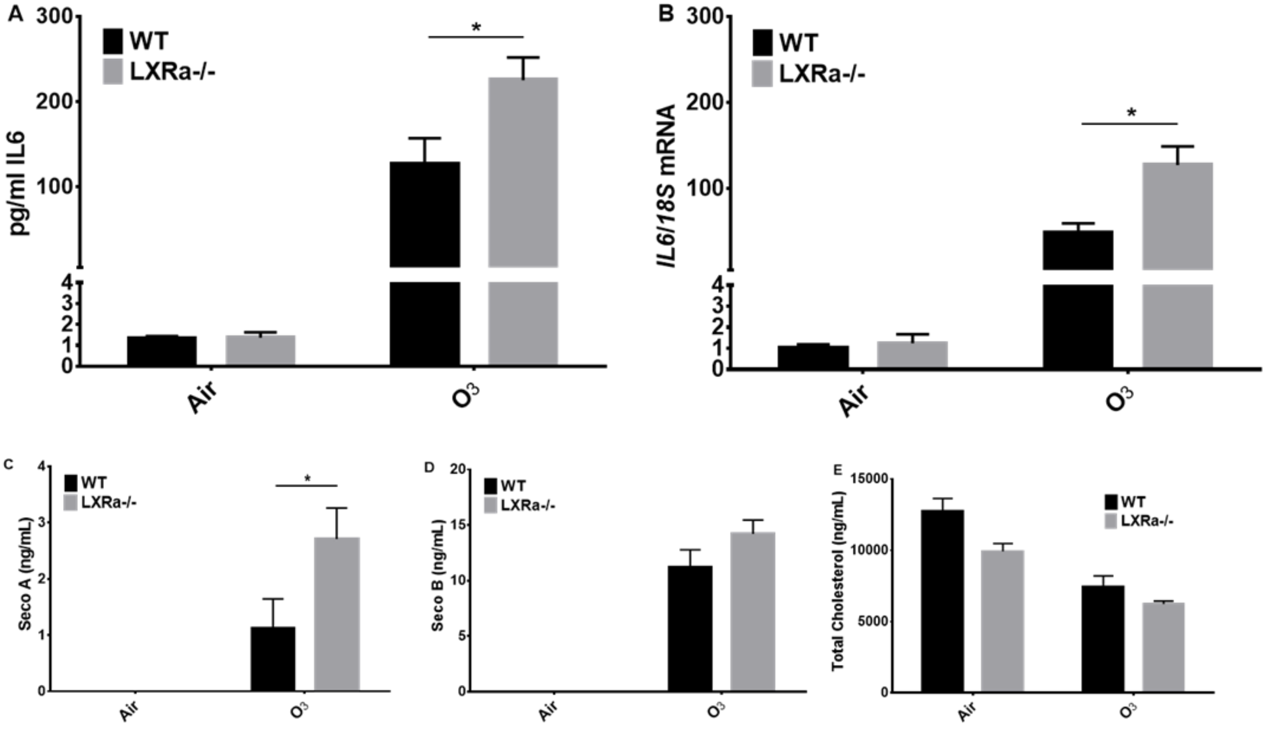
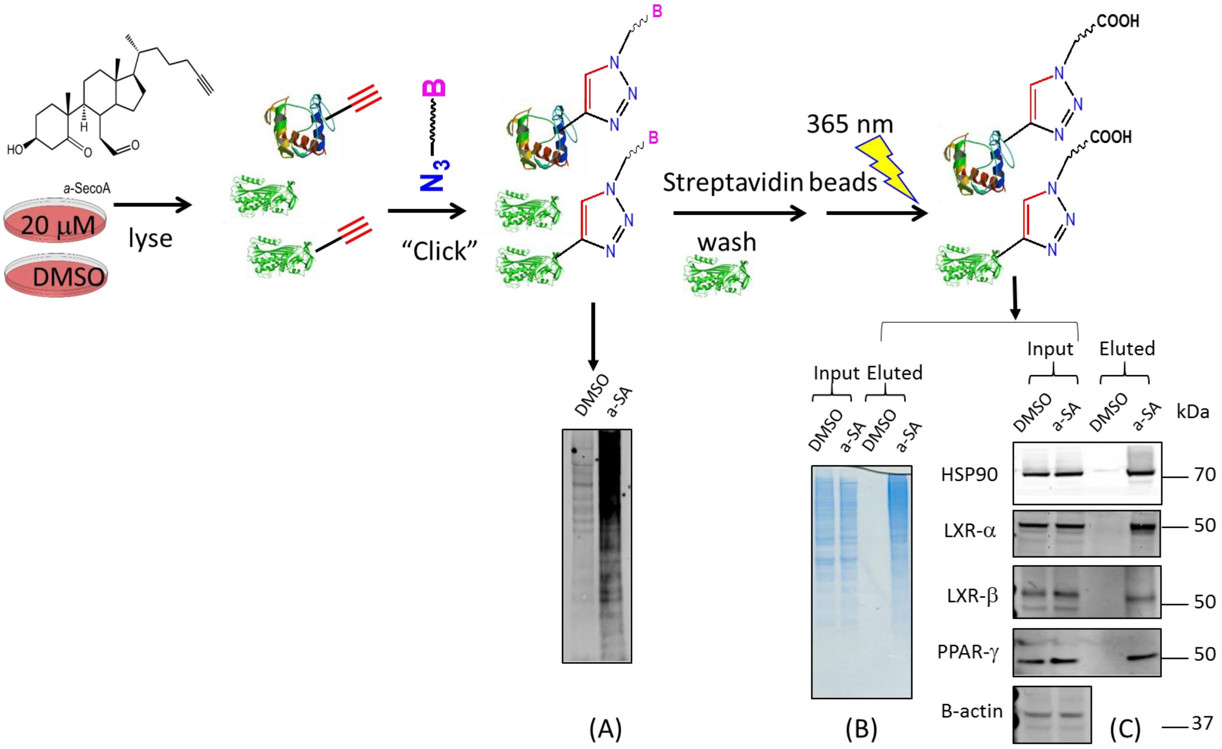


Figure 2.6 Simplified steps of protein catch and photo-release using a-Seco A probe in 16 HBE cells. Supplementation with a-Seco A (20 µM) in 16HBE cells for 4 h at 37 °C followed by cell lysis and click reaction to introduce biotin for immuno affinity purification. A single aliquot was taken to probe the extent of alkyne labeling via biotin-streptavidin interaction. The rest of clicked cells were immobilized onto Streptavidin beads to catch only adducted proteins and remove un-adducted proteins. Subsequent photo-release permitted to collect adducted proteins. (A) IRDye® 800CW Streptavidin visualization exhibits the extent of protein labeling with a-Seco A. (B) SDS Page gel of photo-released proteins. An equal amount of total protein was loaded. The a-Seco A treated cells (right 2 lanes) exhibited significant protein adduction compared to the input lanes (left 2 lanes). (C) Selective antibody analyses indicate adduction on HSP90, LXR-α, LXR-β, and PPAR-γ proteins. Anti-actin of the input lane provides another confirmation of equal loading. Representative blots from 3 separate experiments (n=3).



HO
H
O
O
a-SecoA
20 µM
DMSO
lyse
N_3—B
"Click"
B
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N
B
Streptavidin beads
wash
365 nm
COOH
COOH
DMSO
a-SA
Input
Eluted
DMSO
a-SA
DMSO
a-SA
Input
Eluted
DMSO
a-SA
DMSO
a-SA
kDa
HSP90
70
LXR-α
50
LXR-β
50
PPAR-γ
50
B-actin
37
(A)
(B)
(C)

Figure 2.7: Protein adduct formation is observable in 20 µM a-Chol supplemented 16HBE cells exposed to O₃. Following a-Chol supplementation (20 µM), apical medium was removed and the cells were exposed to clean air or 0.4 ppm O₃ for 4 h. Cells were harvested after 1 h post-incubation (A) Lipids were extracted as described in experiment. LC-SRM profile demonstrates that a-cholesterol was incorporated into the cells and generated ozone derived oxysterols as those of endogenous cholesterol. (B) Schematic depicting a-Chol supplementation prior to O₃ exposure. (C) Cells harvested in lysis buffer containing inhibitors were clicked with azido-biotin. IRDye® 800CW streptavidin probe displays significantly extent of protein adduction upon O₃-exposed cells where as background level of adduction with air-exposed cells were observed. Anti-Actin is served as a loading control. (D) HSP90 and LXR-α westerns of click and photo-released proteins. 16HBE cell lysates were loaded as a positive control. (E) Relative densitometry analysis of LXR-α protein levels from 3 separate immunoblots (n=3).

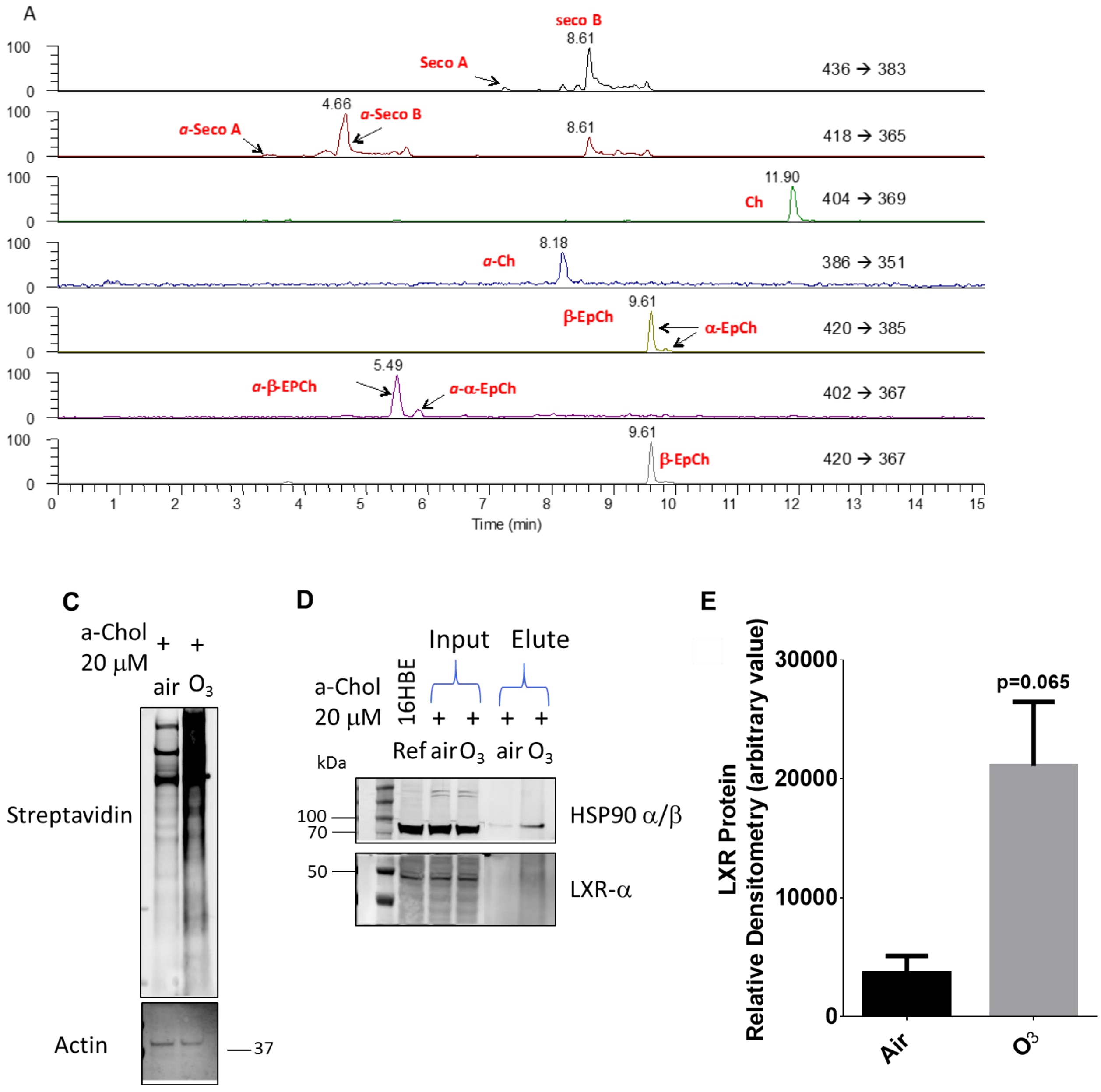


Figure 2.8: Seco A altered cholesterol efflux pump gene expression when treated with Seco A and the T09 LXR agonist in sequence. (A) Experimental design depicting how 16HBE cells were challenged for 2 h to 20 μM of Seco A, 10 μM T09, or DMSO control for first challenge, media removed and followed by 2 h of second challenge. Samples were evaluated for (B) *ABCA1*, (C) *FASN*, and (D) *SREBP1* gene expression. LXRE activity was measured in relative light units compared to the respective vehicle control in the (E) individual oxysterol challenges at 20μM for 4h and (F) sequential treatment in 16HBE cells. Data are presented as mean ± SEM of fold change compared to the DMSO control. Statistical analysis was performed with a one way ANOVA and Fisher's LSD post hoc comparison test, *significantly different from the DMSO control, $^{\&}$significantly different from the indicated conditions, p<0.05, n=3.

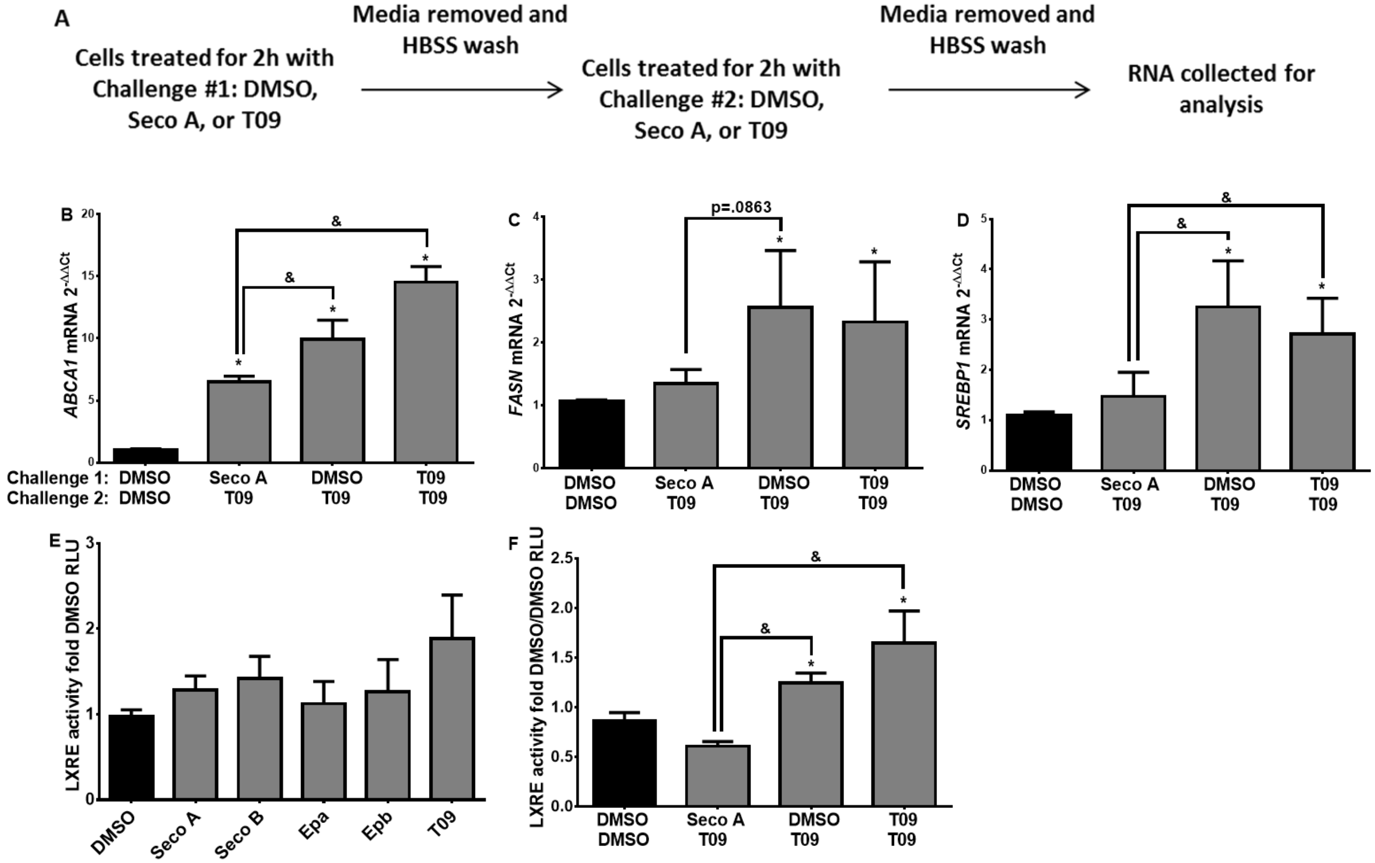
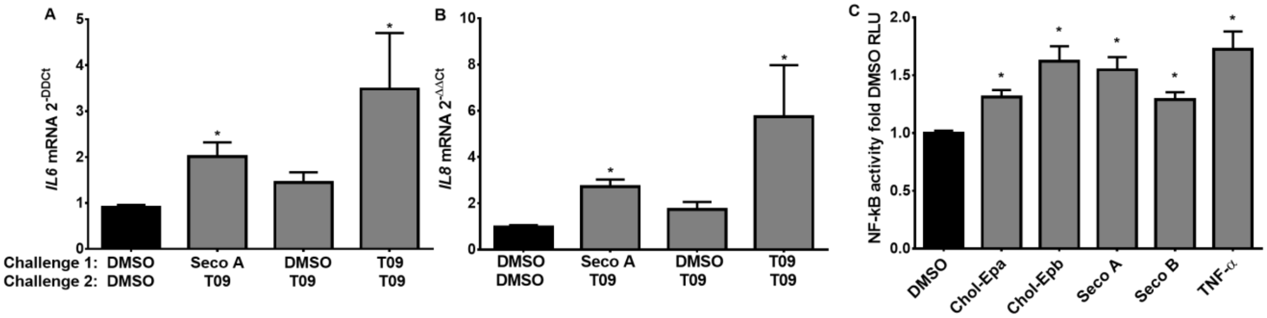


Figure 2.9: Oxysterol altered inflammatory signaling. A) *IL6* and B) *IL8* gene expression in sequential challenge of 2 h with first challenge followed by another 2 h of second challenge on 16HBE cells exposed to 20 µM of Seco A, 10 µM T09, or DMSO control on collagen coated plates. C) Relative light units of NF-κB promoter reporter activity in 16HBE cells exposed to 20 µM of various oxysterols, 20 ng/mL TNF-α, or DMSO negative control for 4 h on collagen coated plastic. Data are presented as mean ± SEM of fold change compared to the DMSO control. Statistical analysis was performed with a one way ANOVA and Fisher's LSD post hoc comparison test, **p*<0.05 n=3



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CHAPTER 3: OZONE-DERIVED OXYSTEROL-PROTEIN ADDUCTS MODIFY NLRP2 INAIRWAY EPITHELIAL CELLS

3.1 Overview

Despite the abundance of studies examining the adverse health effects of ozone (O_3) exposure and its association with increased airway inflammation, the mechanisms mediating these responses have yet to be fully described. Previous studies, including our own, have shown that exposure to O_3 results in the formation of electrophilic lipid peroxidation products, notably through the oxidation of cholesterol in epithelial cells. These electrophilic oxysterols are capable of forming covalent linkages with nucleophilic functional groups on proteins, particularly lysine residues, thus potentially altering cellular signaling pathways. Much remains to be learned about the identity of the reactive lipid species and the range of potential proteins modified by the oxysterols in the lung, as well as the biological consequences. In this study, airway epithelial cells were exposed to the O_3-derived oxysterol alkynyl Secosterol-A and 2D LC-MS/MS shotgun proteomics was used to generate a database of oxysterol-protein adducts. NLR Family Pyrin Domain Containing Protein 2 (NLRP2) was among oxysterol-adducted proteins and bio-orthogonal cycloaddition was used to confirm oxysterol-NLRP2 adduct formation to lysine K1019 in the LRR protein region, a region known to influence inflammasome complex formation. Utilizing qPCR and western blotting, abundance of NLRP2 in airway epithelial cells was compared with other prominently studied NLR proteins. In addition, O_3-induced expression of NLRP2 and association with other inflammasome proteins was tested. To determine the role of NLRP2 in O_3-induced inflammation, we knocked down NLRP2 expression in human airway epithelial cells followed by analysis of inflammasome complex formation and inflammatory mediator production. Our data show that NLRP2 is the most abundantly expressed member of the NLRP family in human airway epithelial cells. Oxysterol-NLRP2 adduction occurred in the leucine-rich-repeat region, which regulates the activity in other NLRP-inflammasome complexes. We also show that exposure of epithelial cells to O_3 further increases NLRP2 expression, activates caspase-1 cleavage, and enhances markers of inflammasome complex assembly, suggesting that O_3 leads to the formation and activation of NLRP2 inflammasome complexes. Knockdown of NLRP2 alone reduces O_3-induced inflammation and we can recapitulate this reduction in inflammasome cytokine signaling by impeding downstream NLRP2 IL-1β and IL-18 signaling with targeted caspase-1 and IL-1 receptor inhibition. Together our findings further support the notion that oxysterol-NLRP2 adduct formation could be mediating O_3-induced inflammation through a decrease in mature IL-1β and IL-18 production. We show for the first time that NLRP2 is highly abundant in airway epithelial cells and a key component of O_3-induced inflammation. Our data further demonstrate that formation of oxysterol-protein adducts with NLRP2 is a novel mechanism mediating O_3-induced pro-inflammatory responses.

3.2 Introduction

Numerous *in vitro* and *in vivo* studies have demonstrated that exposure to O_3 causes rapid activation of the NF-κB signaling pathway, generating pro inflammatory mediators and leading to the infiltration of inflammatory cells into the airways (1-4). Yet, mechanisms linking the known chemical reactivity of O_3 and its reaction products with biological effects continue to present a knowledge gap.

Studies have shown that O_3 reacts directly with sulfhydryl groups in proteins and small molecules, such as ascorbate, glutathione, and urate, and has the ability to form ozonides with unsaturated lipids such as phosphatidylcholines and cholesterol which are abundantly present in the airway surface liquid (5). O_3-derived products of lipid oxidation, including electrophiles such as lipid ozonization products (LOPs) and oxysterols, are capable of independently forming protein adducts and altering normal cellular function (6). A series of studies in the field of O_3-induced lipid peroxidation suggested that LOPs alone could recapitulate O_3-induced activation of NF-κB and pro-inflammatory mediator production (7,8). Similarly, 4-hydroxynonenal (4-HNE), formed during phospholipid peroxidation, causes covalent modification of proteins and also mimics O_3-induced biological effects (9,10). Moreover, our research team's recent studies have focused on the formation of O_3-derived oxysterols (11-13). While their presence has been measured in the lung and in atherosclerotic plaques, few studies have been conducted examining their impact on inflammatory response in the human airway following O_3 exposure.

In the human lung, enzymatically generated oxysterols formed during normal cholesterol metabolism perform anti-inflammatory and immune-modulatory functions (14). In contrast, oxysterols formed via ozonolysis of cholesterol are highly electrophilic and lead to the formation of covalent linkages with proteins, modifying normal function (13). Rodent studies and our own human *in vivo* and *in vitro* exposure studies demonstrate that O_3 exposure increases the abundance of several electrophilic oxysterol species in epithelial cells and airway surface liquid and that these oxysterols can increase NF-κB activity in airway epithelial cells (6,15). In addition, our previous studies indicate that while endogenously formed oxysterols activate the liver-X-receptor (LXR) and are anti-inflammatory (16), O_3-derived oxysterols inhibit the LXR pathway and are pro-inflammatory, suggesting opposing functions of endogenously formed and ozonolysis-derived oxysterols (12).

Biochemical analysis indicates that electrophilic O_3-derived oxysterols react with nucleophilic centers of proteins (largely lysine residues), forming oxysterol-protein adducts, which could affect the function of these proteins (13), thus providing a new paradigm linking O_3-induced chemical modification of lipids with changes in protein function. As depicted in Figure 3.1 and discussed previously in Chapter 2, our previous data demonstrated O_3-induced generation of oxysterols, such as Secosterol A (Seco A), in humans *in vivo* and epithelial cells *in vitro* and are capable of forming protein adducts (12). Based on this study we designed experiments to conduct an unbiased assessment of the overall “adductome”. Specifically, we developed experimental protocols in which airway epithelial cells were treated with alkynyl-modified O_3-derived oxysterols, followed by reaction of cell lysates with an azido biotin reagent under “click” cycloaddition conditions. This sequence of reactions results in the biotinylation of any protein that forms a covalent bond with *a*-Seco A (17). The immunoprecipitated and photo-eluted fraction was analyzed for the overall “adductome” in epithelial cells using 2D LC-MS/MS shotgun proteomics, thus identifying potentially novel targets for O_3-derived oxysterols. The findings detailed in this study identify NLRP2 as a highly abundant oxysterol-adducted protein, which regulates O_3-induced inflammatory responses in epithelial cells, thus uncovering a novel signaling mechanism regulating the inflammatory effects caused by O_3 exposure in the human airway. Our study demonstrates that assessment of the O_3-derived oxysterol “adductome” in human airway epithelial cells has the potential to uncover novel cellular targets and mechanisms mediating O_3 toxicity.

3.3 Materials and Methods

Reagents - Alkynyl-probes were synthesized following the procedures published previously (2,13,18). DTT and iodoacetamide were purchased from Sigma Aldrich (St Louise, MO). Streptavidin beads were purchased from GE Healthcare (Piscataway, NJ). The following reagents were purchased from their respective companies: 10% NuPage Novex Bis-Tris® precast mini gel (Invitrogen, Grand Island, NY), PVDF membrane and Simply Blue (Bio Rad, Hercules, CA), IRDye® 800CW Streptavidin (925-32230, Li-Cor, Lincoln, NE), Blocking buffer (Rockland, Gillbertsville, PA or Odyssey Blocking buffer, Li-Cor, NE). Sequencing grade Trypsin (Promega V5111, Madison, WI). NLRP2 antibody was obtained from R&D Systems (Minneapolis, MN) Antibodies of NLRP3, NLRP1, and HIS3 were obtained from Cell Signal Technologies (Danvers, MA), ASC and Actin were from Santa Cruz (Dallas, Texas). Cell Culture - 16HBE14o (16HBE) cells, a SV-40 transformed human bronchial epithelial cell line were a gift from Dr. D. C. Gruenert (University of California San Francisco, San Francisco, CA). 16HBE cells were plated on fibronectin-coated (LHC Basal Medium [Life Technologies, Carlsbad, CA], 0.01% BSA [Sigma, St. Louis, MO], 1% Vitricol [Advanced Bio Matrix, San Diego, CA], and 1% human fibronectin [BD Biosciences, San Jose, CA]) 0.4µm Transwell® plates(Costar, Corning, NY), and grown submerged in minimal essential media (Gibco) with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine (Life Technologies) until confluent for 6 days, and 1 day at air-liquid interface (ALI) before use. Primary human bronchial epithelial cells (HBEC) were obtained from healthy donors in collaboration with the Environmental Protection Agency (EPA) using a protocol approved by the University of North Carolina at Chapel Hill Institutional Review Board (Chapel Hill, NC), as described previously (19). The HBECs were cultured in Pneumacult medium for 21 days to differentiate according to previously described methods (20,21).

In Vitro O_3 *Exposure* - Cultures at ALI were exposed to filtered air or 0.4ppm O_3 for 4 hours in exposure chambers operated by the U.S. EPA, as previously described (22). The dose was selected for maximal innate immune response to O_3 with minimal cytotoxicity and has been used previously by our group (21). At 1 or 24 h after exposure, apical sides of all cultures were washed with 100 µL Hanks' balanced salt solution (HBSS) (Life Technologies) and saved for LC-MS analysis. The remaining cells were collected in 200 µL PBS, centrifuged at 500 g for 5 min, and were stored at -80 °C until LC-MS analysis.

Real-time qPCR - Total RNA was isolated from 16HBEs and HBECs with the use of the Pure Link RNA Mini Kit (Life Technologies, Carlsbad, CA). First strand cDNA preparation and real-time quantitative PCR (qPCR) were performed as previously described (23,24). Primers and probes for NLRP1, NLRP2, NLRP3, NLRP7, NLRP12, and β-actin were commercially available (Applied Biosystems, Foster City, CA). Human IL8: 5'-FAM-CCTTGGCAAAACTGCACCTTCAC-TAMRA-3' (probe), 5'-TTGGCAGCCTTCCTGATTTC-3' (sense), and 5'-TATGCACTGACATCTAAGTTCTTTAGCA-3' (antisense) and IL6: 5'-FAM-CCAGCATCAGTCCCAAGAAGGCAACT-TAMRA-3' (probe), 5'-TATGAAGTTCCTCTCTGCAAGAGA-3' (sense), and 5'-TAGGGAAGGCCGTGGTT-3' (antisense) were prepared in-house. Differences in expression were determined with the ΔΔCt method and β-actin for normalization. Threshold cycle (Ct) value for the housekeeping gene (β-actin) was subtracted from the Ct value for the gene of interest to determine the ΔCt value. For each pairwise set of samples to be compared, the difference in ΔCt values between the two samples were calculated for the genes of interest to determine the ΔCt value. The fold change in gene expression was calculated as 2-ΔΔCt.

Sterol profiling using PTAD derivatization and LC-SRM Analysis. Cells were scraped into 400μL cold PBS. 200 of the 400 μL were taken and internal standards added (13 ng for d7-7-DHC, 97 ng for 13C3-Desmosterol and 99 ng for 13C3-Lanosterol, and 342 ng for d7-Cholesterol/sample), 10 μL butylated hydroxytoluene (BHT)/triphenylphosphine (TPP) solution (2.5 mg TPP and 1 mg BHT in 1 mL MeOH), 400 μL 1% NaCl, and 500 μL Folch solution (2:1=CHCl3:MeOH). Cells and standards were vortexed vigorously and centrifuged at 3099 x g for 5 min. CHCl3 layer was removed and added to PTAD-predeposited tube (200 μg/tube). The sample tubes were vortexed and analyzed by LC-MS using the following conditions: 10 μL was injected onto the column (Acquity UPLC BEH C18, 1.7 μm, 2.1 x 50 mm) with 100% MeOH (0.1% acetic acid) mobile phase for 1 min runtime at a flow rate of 300 μμL/min. The monitored transitions included: 7-DHC m/z 560→365, d7-7-DHC m/z 567→372, desmosterol m/z 592→365, 13C3-desmosterol m/z 595→368, lanosterol m/z 634→602, 13C3-lanosterol m/z 637→605 with retention times of 0.8, 0.5, and 0.6 min, respectively.

Oxysterol Extraction from 16HBE or HBEC cells. - To the cell pellets, 10 μL alkynyl-Cholesterol (*a*-Chol) (25ng/μL), 500 μL NaCl (0.9%), 10 μL of TPP and BHT (25 mg TPP and 10 mg BHT in 10 mL MeOH), 1 mL of Folch solution (2:1=CHCl3:MeOH) were added. The mixture was mixed vigorously by vortex for 2 min and separated by centrifugation with 2300 x g for 3 min. The collected organic layer (bottom layer) was evaporated to dryness in a SpeedVac™ concentrator and resuspended in 100 μL MeOH for LC-MS analysis.

LC-MS Analysis - The resuspended samples were chromatographed by RP-HPLC using a UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm) in Waters Acquity UPLC system equipped with an autosampler (Waters, Milford, MA) and either ESI or APCI in positive ion mode. For

ESI, the oxysterols were separated by 95% solvent B in an isocratic method with a flow rate of 200 L/min, and the mobile phase solvents consisted of 2 mM NH4OAc (solvent A) in water and 2 mM NH4OAc in MeOH (solvent B). The injection volume was 10 μL using a partial loop with needle overfill mode. MS detections were done using a TSQ Quantum Ultra tandem mass spectrometer (ThermoFisher, Waltham, MA), and data was acquired and analyzed using a Thermo Xcalibur™ 2.2 software package. The cholesterol and oxysterols form [M+NH4]+ ions in positive ion mode. The transitions monitored were m/z 436→383 for Seco A/B, m/z 418→365 for *a*-Seco A/B, m/z 404→369 for cholesterol (Chol), m/z 386→351 for a-Chol, m/z 420→385 for EpCh, m/z 402→367 for a-EpCh. For APCI, 95% MeOH in H2O containing 0.01% acetic acid was used as a mobile phase. The cholesterol and oxysterols form [M+H]+ ions in positive ion mode. The transitions were m/z 369→369 for Chol, m/z 383→383 for Seco A/B, m/z 351→351 for a-Chol, m/z 385→385 for EpCh. The transitions of cholesterol and cholesterol esters were m/z 365 →365 monitored by HPLC-MS following the method described elsewhere (25). The amount of the cholesterol esters was found to be less than 2% of free cholesterol in the cells studied.

Protein Cytokine Analysis: Concentration of IL-6 and IL-8 in the collected basolateral media of 16HBE cells exposed to O_3 was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions (BD Biosciences, San Jose, CA).

Immunoprecipitation and Western Blotting: Cell lysates from 16HBE cells exposed to air/ O_3 or O_3 derived oxysterols were separated by 10% SDS-PAGE and transferred to nitrocellulose. Proteins were detected using specific antibodies: ASC (1:500), NLRP1 (1:1000), NLRP2 (1μg/ml), NLRP3 (1:1000), or β-actin (1-2000), which served as a loading control. Antigen-antibody complexes were incubated with horseradish peroxidase-conjugated secondary antibody and were detected using chemiluminescence. Immunoprecipitation of NLRP2 bound ASC was performed using 1mg of whole cell lysate incubated overnight with NLRP2 antibody at 4°C followed by addition of an equal volume of Protein A agarose beads (Thermo Scientific 20333) and incubation at RT for 2 h. The agarose bound protein was washed with 500µL IP buffer (25mM Tris, 150mM NaCl; pH 7.2) 3 times and washed once with 500µL ddH_2O. The protein was eluted by adding 40µL of 5X Loading Buffer with 2-mercaptoethanol (Sigma M3148) and heated to 95°C for 5 minutes. The samples was then separated on a 10% SDS-PAGE gel and transferred to nitrocellulose (BioRad 1620070). Proteins were detected using specific antibodies noted above and antigen-antibody complexes were incubated with horseradish peroxidase-conjugated secondary and detected using chemiluminescence.

Cell Culture and Whole Cell Labeling with a-Seco A in 16HBE - 16HBE cells were plated 2 x 106 in 10 cm plates using the conditions described above, then allowed to settle and grow for 24 h. The cells were then incubated in the presence of *a*-Seco A (20 µM) and reduced FBS levels (2%) in MEM medium for 4 h. Cells were harvested in lysis buffer (50 mM HEPES, 150 mM NaCl, 0.1% TritonX100, pH=7.0) containing protease inhibitors (Sigma P8340) on ice. The lysate was cleared by centrifugation at 10,000 x g for 10 min @ 4 °C to remove cellular debris. The total protein concentration was determined using standard BCA assay (Pierce, ThermoFisher) and utilized for click reaction assay.

Cell Culture and Whole Cell Labeling with a-Cholesterol Followed by Ozone Exposure in Human Bronchial Epithelial Cells (16HBE) - 16HBE cells were grown in 24 mm transwells submerged in MEM with reduced FBS containing 20 µM *a*-Chol for 6 days. The apical media was removed 24 h before exposure. Next, the plate was exposed to filtered air or O_3 (0.4 ppm) for 4 h and allowed to continue incubating for an additional 1 h without O_3 (1 h post incubation).

Cells were harvested in 300 uL lysis buffer/well and analyzed as described above.

Click Biotinylation of a-Seco A/a-oxysterols Adducted Proteins in 16HBE and Streptavidin Affinity Purification - Our previous studies with a model protein (13) and 16HBE cell line (12) has shown rapid modification of proteins using an alkynyl tagged alkynyl-secosterol A (*a*-Seco A) and "click cycloaddition methods to capture modified proteins. This approach makes generating an inventory of the total "secosterol-adductomes" in EC possible by the workflow shown in Figure 3.2. In short, *a*-Seco A treated cells were lysed and "click" reacted with a photo-cleavable azido-biotin. Streptavidin visualization from a small portion of cell lysate showed a host of cellular proteins adducted by *a*-SecoA. After streptavidin confirmation of adduct formation, the remaining lysate was precipitated in cold MeOH and excess click reagents were removed. The precipitated and washed cell pellets were re-suspended in PBS in the presence of streptavidin beads to pull down the adducted proteins and after 2 h, the beads were washed sequentially with SDS, urea, NaCl, and ammonium bicarbonate to remove the unbound, and therefore un-adducted, proteins. The washed beads were exposed to a small portable UV lamp (365 nm) for 2 h to release the adducted proteins and the freed protein adducts were dried and then re-suspended in LDS buffer followed by a short stacking SDS-PAGE run. The blue stain shows that an array of proteins with a broad range of molecular weights had formed adducts with *a*-SecoA. The stained gels were excised followed by in-gel trypsin digestion and the digested peptides were extracted from the gel, dried, and reconstituted for MudPIT LC-MS/MS analysis.

Visualization of Biotinylated Proteins Adducted with a-SecoA/a-Oxysterols - The saved 50uL of click-reaction mixture was mixed with SDS sample loading buffer and resolved using 10% NuPAGE Novex BisTris® gel (Invitrogen, Carlsbad, CA). The proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules CA) and probed with IRDye® 800CW Streptavidin (Li-Cor, Lincoln, NE). The extent of adduction was visualized using Odyssey Infrared Imaging System™.

Immunoblotting Analysis of Photo-released Proteins - The photo-released and dried proteins were reconstituted in 70µL PBS, 25µL LDS buffer, and 5µL of 1M DTT and resolved by 10% NuPAGE Novex BisTris® gel then transferred to PVDF membrane. The transferred proteins were incubated with antibodies of NLRP2 (anti-mouse, R&D), Histone H3 (anti-mouse, Cell Signaling), overnight in the cold room at 4 °C. Alexa Fluor 680® labeled secondary anti-mouse was used to detect target proteins. Immunoreactive proteins were visualized using Odyssey Infrared Imaging System™ (Li-Cor, Lincoln, NE).

Anakinra/Cas1 Inhibition of inflammatory signaling in 16HBE cells – As described above, 16HBE cells were cultured at ALI until formation of a monolayer prior to treatment or exposure. Anakinra (rhIL-1ra) (R&D Systems 280-RA) at 500ng/mL and 1µg/mL and Caspase 1 inhibitor N-CBZ-Trp-Glu(OMe-His-Asp(OMe) fluoromethyl ketone (Sigma C-1855) at 10 and 20µM were added to the basolateral compartment in 2%FBS MEM media for 24 h. The next day the treatments were refreshed and the cells were exposed to 0.4ppm O_3 for 4 h. total RNA was collected 1 h post exposure.

TRIPZ shRNA NLRP2 knockdown in 16HBE cells – NLRP2 shRNA was purchased from Life Technologies Dharmacon (RHS5087-EG55655). The vector was grown up in competent cells and isolated by the UNC vector core. The lentiviral vectors were added into 16HBE and selected for by puromycin at 1µg/mL prior to being maintained or frozen down in LN2. 24 h prior to any treatment or exposure the shNLRP2 was activated by 1µg/mL doxycycline and cells were treated according to the desired experimental protocol.

Statistical Analysis- All *in vitro* data were performed in at least 3 separate experiments, each with multiple technical replicates. Data shown are mean ± SEM. See figure legends for further information on the specific statistical analysis used for each experiment.

3.4 Results

Characterizing the O_3*-derived oxysterol adductome in 16HBE cells using alkynyl tagged Secosterol A (a-SecoA)* - In this Chapter, we show that exposure to O_3 results in the formation of electrophilic oxysterols, which are capable of forming covalent linkages with nucleophilic sites in proteins, particularly in lysine residues. While a number of studies, including our own (see Chapter 2) demonstrate modification of specific proteins with cholesterol ozonolysis products, unbiased analysis of the O_3-derived oxysterol-adductome could yield novel targets for understanding O_3–induced inflammation. The workflow shown in Figure 3.2 shows the shotgun proteomics using a tagged *alkynyl*-secosterolA (*a*-SecoA) and “click cycloaddition” assay, revealing the total inventory of “secosterol-adductomes” in epithelial cells. Streptavidin visualization from a small portion of cell lysate showed a host of cellular proteins adducted by *a*-SecoA. (Figure 3.2). After streptavidin confirmation of protein adduct formation, the remaining lysate was precipitated in cold MeOH to remove excess click reagents. The precipitated and washed cell pellets were re-suspended in PBS in the presence of streptavidin beads to pull down the adducted proteins and after 2 h, the beads were washed sequentially with SDS, urea, NaCl, and ammonium bicarbonate to remove the unbound, and therefore un-adducted, proteins.

The washed beads were exposed to a small UV lamp (365 nm) for 2 h to release the adducted proteins and the freed protein adducts were dried and then re-suspended in LDS buffer followed by a short stacking SDS-PAGE run. The blue stain shows that an array of proteins with a broad range of molecular weights had formed adducts with *a*-SecoA. The stained gels were excised followed by in-gel trypsin digestion and the digested peptides were extracted from the gel, dried, and reconstituted for MudPIT LC-MS/MS analysis. Experiments were carried out in triplicate. RAW data were searched using MS-GF+beta (V9517) against humanRefSeq_Version54 database (Sep 25, 2012; 34589 proteins). Precursor ion mass tolerance was 10 ppm, and fragmentation tolerance was 0.01 Da for the database search. The maximum number of modifications of 15.9949 Da (Methionine oxidation), + 57.0215 Da (Carbamidomethyl alkylation to Cys), + 541.3880 (*a*-SecoA-triazol-hexanoic acid modification to Lys) were searched as dynamic modifications. The maximum Q value of peptide-spectrum matches was set as 0.01 using IDPicker software (V. 3.1.9288) that includes 2 minimum spectra per protein, and 1 minimum additional peptide. The IDPicker report was assembled by the mass shift 541.3880 Da from the *a*-SecoA modification shown the structure in Figure 3.3. We have observed that the *a*-SecoA modified peptides produced diagnostic fragments (DF1 and DF2) m/z 512 and 129 respectively shown in Figure 3.3. To ensure the accuracy of the identification, all modified peptides were manually inspected with the precursor ion accuracy within 10 ppm. After manual inspection, 130 unique proteins were recorded to be adducted by *a*-SecoA. Of these 27 were associated with cell cycle, 28 with cytoskeletal, 30 with structural proteins, 28 with chaperone proteins, and 14 were unique functional proteins. The adducted protein association network is shown in Figure 3.5 and the 14 unique functional proteins adducted with SecoA are listed in Table 3.1.

NLR family protein adduction by O_3 *derived oxysterol SecoA* - Of the proteins listed in Table 3.1, NLRP2 stands out as the sole adducted protein known to be associated with damage associated molecular patterns like O_3. In fact, previous studies have identified patterns of inflammasome activity following O_3 exposure, primarily driven by NALP family proteins (26,27). Of the NALP family proteins, only NLRP2 (NACHT, LRR and PYD domains-containing Protein 2) was found to be modified by *a*-SecoA. A total of 57 peptides belonging to NLRP2 (54% coverage) were identified with 218 spectral counts (Figure 3.4C). Lys1019 in the sequence of LK*IDDFNDELNK was modified by *a*-SecoA precursor found m/z 669.0840 (µm 1.1 ppm) (Figure 3.4A). The b and y ions including the adducted Lys residue are well matched to this adduction site. Further the DF1 and DF2 also confirm the modification by SecoA (Figure 3.4B).

NLRP2 abundance in various human airway cells - In order to understand the relative abundance of NLR family proteins in various primary human airway cells and cell lines, we examined the mRNA levels and relative protein abundance of five additional NLR family members. Our primary human bronchial epithelial cells (HBECs) as well as human airway epithelial cell lines (16HBE, B2B, A549) exhibited significantly higher levels of NLRP2 mRNA (Fig. 3.6A-F) and protein (Fig. 3.6G) compared to the other NLR family members measured. THP-1 cells, a human blood monocyte cell line, and the K562 cells, a blastoma cell line, are known to have high levels of NLRP3 and NLRP1 respectively (28). Our results reflect this characterization as the THP-1 cells have significantly more NLRP3 protein and mRNA signal while the K562 cells exhibit significantly higher levels of NLRP1 compared to the other measured targets. Since NLRP2 is one of the least understood members of the NLR inflammasome complex proteins, we confirmed expression and abundance of this protein in a variety of cells and tissue. We show for the first time, the high abundance of NLRP2 in human primary airway epithelial cells and cell lines, and recapitulate the lower abundance in both monocytic and lymphocytic cell lines previously shown (29,30).

Confirmation of a-Seco A NLRP2 Adduction by Immunoblotting - Shotgun proteomics data revealed that Seco A, a primary ozonide, formed protein adducts with NLRP2. In order to validate this finding by targeted immunoblotting assay, *a*-SecoA was used to “catch and release the adducted proteins” as shown in Figure 3.2. In this model, the immunoaffinity purified protein with streptavidin beads and photo-released protein mixture were subsequently subjected to western blot analyses using antibodies against Histone 3 (HIS3), NLRP2, and NLRP3 (Fig. 3.7). HIS 3 is utilized as a known positive control for SecoA adduction, it is a highly conserved abundant histone protein subunit that also appears at the top of our list of adducted proteins. Input lanes represent the click reaction mixtures and show the equal amount of loaded protein between the vehicle control and *a*-SecoA treated sample. The photoreleased lanes reveal the *a*-SecoA adduction of NLRP2 and HIS3 in *a*-SecoA treated cells and only insignificant amounts of proteins in the vehicle control treated cells due to non-specific binding to the Streptavidin beads. No such SecoA-NLRP3 adducts were observed in 16HBE cells. For reference, we also tested NLRP2 and NLRP3 adduction in THP-1 cells, a monocyte cell line previously reported to express high levels of NLRP3 (28). While NLRP3 is present in the THP-1 cells, there is no or limited observed adduction by Seco A (Fig 3.7A/E).

Alkynyl Cholesterol Supplemented Cells Exposed to O_3 *Reveals the Formation of NLRP2-adducts* - 16HBE cells were grown in the presence of 20μM alkynyl-tagged cholesterol (*a*-Chol) for 6 days. Media containing *a*-Chol was refreshed daily and the apical side was washed 24h prior to O_3 exposure. 16HBE cells were exposed to filtered air or 0.4ppm O_3 for 4 h and harvested 1 h post-exposure. We have previously shown that *a*-Chol, a surrogate of endogenous cholesterol, is incorporated into the cells and, in response to O_3, generates a host of alkynyl-oxysterols identical to the oxysterol species generated via oxidation of endogenous cholesterol. (12) The streptavidin visualization shows that *a*-Chol incorporated 16HBE cells followed by O_3 exposure exhibit a higher level of overall protein adduction than the air-exposed controls (Fig 3.7B). Photoreleased proteins were resolved in SDS-PAGE and transferred to nitrocellulose membrane and probed with antibodies of NLRP2 and HIS3. Similar to data seen in cells treated with a-SecoA shown in Figure 3.7A, NLRP2 and HIS3 were found to be targets of alkynyl-tagged oxysterols generated endogenously in cells exposed to O_3 (Fig 3.7B).

NLRP2 and Inflammasome Activation in Human Airway Tissues - In order to further support our observed prevalence of NLRP2 in human airway epithelial cells, we conducted immunohistochemistry analysis for NLRP2 protein and its association with inflammasome complex adaptor protein apoptosis associated speck-like protein containing a CARD (ASC) (31). Western blot analysis confirms that NLRP2 protein and mRNA levels (Fig. 3.8A,B), as well caspase-1 cleavage products are significantly increased in 16HBE cells exposed to O_3 compared to filtered air (Fig. 3.8B). In 16HBE cells, NLRP2 functions similar to its better known NLR family proteins by interacting with caspase-1 activity via the adaptor protein ASC, forming an inflammasome complex in order to produce cleaved IL-1β and IL-18. Figure 3.8C shows that exposure of 16HBE cells to 0.4ppm O_3 for 4 h increased expression of NLRP2 protein and ASC independently when compared to cells exposed to filtered air. In the final panel of Figure 3.8A, an overlay of the two targets shows an increase in association between NLRP2 and ASC following O_3 exposure, indicating both that O_3 exposure increases inflammasome signaling in human airway epithelial cells and that NLRP2-ASC association is a key step in the process.

NLRP2 Knockdown decreases Inflammatory Signaling - To determine the role of NLRP2 in O_3-induced inflammation, we used lentiviral transduction of NLPR2-specific shRNA to knock down NLRP2 expression in 16HBE cells. The TRIPZ inducible lentiviral shRNA allows the vector to be induced specifically in the presence of doxycycline. Our experiments include a control exposure of cells that are transduced but the vector remains uninduced with doxycycline. Figure 3.9 shows our lentiviral transduction of NLRP2 shRNA significantly reduced NLRP2 mRNA levels (Fig 3.9A) and protein levels (Fig. 3.9B) to less than 10% of control 16HBE cells. In addition, knocking down NLRP2 in 16HBE cells also significantly reduced O_3 induced markers of inflammation, *IL-6* (Fig. 3.10A) and *IL-8* (Fig 3.10B) by almost 75% of control 16HBE cells. These data suggest that reduced NLRP2 in 16HBE cells inhibits NLRP2 downstream activity necessary for inflammasome activity as well as inhibits the O_3 induced pro-inflammatory signaling response.

Caspase-1 and IL-1aR inhibitors Blunt O_3 *Induced Markers of Inflammation* - IL-1β and IL-18 protein levels in the apical washes or basolateral supernatants were below the limit of detection using traditional ELISA methods (data not shown). Therefore, to examine the role of NLRP2-mediated inflammasome activation and the role of potential autocrine IL-1 signaling in O_3-induced expression of IL-6 and IL-8, we used inhibition of caspase-1 and IL-1 signaling. To do so, 16HBE cells were treated for 24h with the IL1 receptor antagonist Anakinra or the caspase-1 specific inhibitor Z-Trp-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone prior to exposure to 0.4 O_3 or filtered air for 4h. Treatment with both 500ng/mL and 1µg/mL Anakinra produced a dose dependent decrease in *IL-6* (Fig 3.11A) and *IL-8* (Fig 3.11B) expression in the O_3 exposed 16HBE cells. Similarly, treatment with the caspase-1 inhibitor at 20µM and 40µM decreased O_3 induced inflammatory cytokine markers in *IL-6* (Fig. 3.11A) and IL-8 (Fig. 3.11B). Together, these data indicate that caspase-1 and IL-1 dependent signaling mediates O_3-induced expression of *IL-6* and IL-8

3.5 Discussion

Overall, this chapter describes a new paradigm involving NLRP2-inflammasome signaling and O_3-induced inflammation in airway epithelial cells. We utilize a convergence of existing methodologies and previous studies from multiple fields in order to provide new evidence uncovering novel targets of O_3-derived oxysterol modification that mediate the well-described O_3 inflammatory response in the human airway. Our unbiased “adductomics” and protein analysis identified NLRP2 as a newly relevant and highly abundant protein in human airway epithelial cells and a target for O_3-derived oxysterol adduction. Our subsequent knockdown of NLRP2 confirmed its function in inflammasome complex formation and links O_3-induced NLRP2 adduction and O_3-induced inflammation in the lung. In the identified group of adducted target proteins, NLRP2 was identified as the sole inflammasome-signaling target among mainly abundant structural and cytoskeletal proteins. Overall, our findings introduce and delineate a novel signaling pathway linking exposure to O_3 and pro-inflammatory responses in the human airway.

Prior to the study described in this manuscript, there was only minimal research reported into the prevalence and function of NLRP2 as it pertains to human health, with no publications examining its role in the human lung. Our study serves to quantify and describe its role in comparison to other NLR family protein species in the airway. Our screening of five of the most studied NLR protein family members yielded the surprising result as primary human airway epithelial cells and commonly used airway epithelial cell lines presented NLRP2 as the most abundant NLR protein at baseline. We strengthen this novel discovery by comparing NLR family members in THP-1 and K562 cells, which have higher levels of NLRP3 and NLRP1 respectively. This comparison uncovers NLRP2 as a novel target in the human airway epithelium with a potential function that may play important roles in airway inflammasome response to pathogens and pollutants. Based on our findings, O_3-dependent activation of NLRP2 in airway epithelial cells appears to result in the formation of an inflammasome complex in a similar manner to that described for NLRP3, oligomerizing with ASC and pro-caspase 1. (32). Immunohistochemistry analysis in Figure 3.8C shows that expression of NLRP2 is not only enhanced but also associates (co-localizes) with the ASC adaptor protein following O_3 exposure, suggesting increased inflammasome complex formation. Figure 3.8A also demonstrates that O_3 exposure increases caspase-1 cleavage products, additional markers of inflammasome complex formation and downstream caspase-1 signaling. To further examine the link between O_3, inflammasome activation, and pro-inflammatory mediator release, we examined whether the expression of *IL-6* and *IL-8* after O_3 exposure is mediated by intermediary IL-1β release, which functions in an autocrine/paracrine manner to activate pro-inflammatory gene expression. Inhibiting either caspase-1 or the IL-1 receptor also reduces expression of IL-6 and IL-8, revealing a probable link between NLRP2 signaling and inflammatory response. In this context, our evidence corroborates the requirement for inflammasome activity in O_3-induced pro-inflammatory mediator expression, with the NLRP2 inflammasome complex being a novel intermediary step in this process.

Before considering NLRP2's prevalence and function, it is useful to consider NLR family protein function as a whole. Associations between NLR family protein structure, inflammasome activity, and inflammation is mostly conserved and have previously been examined for their interaction in the presence of damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) (33-37). Remarkably, the field of NLR protein biology provides some conflicting evidence on their function in inflammation as conformation state and isoform of the protein determines whether the NLR protein inhibits or agonizes inflammatory signaling (38).

Specifically, studies in the lung show NLR signaling can greatly vary in response to bacterial infection, age related suppression of NLR signaling, and environmental exposures. In patients with compromised immune systems, *A. baumannii* bacteria can activate the host innate NLRP3 pathway accounting for uncontrolled inflammation and lung injury (39). Lungs of aged mice with suppressed NLRP3 expression and decreased IL-1β production are more prone to *S. pneumoniae* infection. Conversely, NLRP6 deficient mice had enhanced NF-κB activity but showed resistance to bacterial infection (40). NLRP3 inhibition also protected mice during infection (41). Downstream, caspase-1 inhibitors decreased IL-1β secretion and reduced the inflammatory effects of cigarette smoke and diesel exhaust in RAW cells, known to have low levels of NLRP2, with upregulated inflammasome activity (29,42). Finally, O_3 exposure is known to lead to NLRP3 inflammasome activation and IL-1 release in rodent models, orchestrating caspase-1 and IL-1 signaling cascade to IL-17A release and neutrophilic airway influx (26,27). Based on these varied responses it is critical to understand the constituency of NLR proteins in the human airway epithelium and their response to pollutants such as O_3.

In terms of NLRP2, previous studies have reported its capacity as an inhibitor of NF-κB activation, enhancing caspase-1 activity in gut, uterine, and immune cells types, however, NLRP2 activity in epithelial cells, in the lung, or in the context of O_3 exposure is completely unknown (29). NLRP2 has been explored as a root cause of inflammasome activity in neuronal astrocytes in the human brain, as knock down of NLRP2 in these astrocytes impaired inflammasome activation leading to reduced caspase 1 activity (43). Furthermore, a nonfunctional allelic variant of murine NLRP2 in the NACHt domain (I352S) generates a protein lacking the NF-κB inhibitory step and exhibits heightened inflammatory stimuli (44). Hence it is plausible that oxysterol-induced modification of NLRP2 modifies its ability to regulate NF-kB signaling, resulting in enhanced inflammation independent of inflammasome complex formation.

NLRP2 adduction by O_3-derived oxysterols exhibits a new paradigm to be studied in the framework of airway inflammation. Many factors impact the sterile inflammation associated with O_3 inhalation exposure, however few concrete mechanisms linking O_3-induced modification of biomolecules, like cholesterol, and cellular outcomes, such as increased pro-inflammatory mediator expression, have been fully established. Our previous studies have ascertained that functional changes to key proteins due to adduction by O_3-derived oxysterols can modify function and increase inflammatory signaling. NLRP2 abundance and its prevalence as a nucleophilic actor in the airway epithelium make it a potentially important target for oxysterol adduction and functional modification. In order to study NLRP2 in human airway epithelial cells, our analysis combines bioorthagonal cycloaddition chemistry with LC-MS/MS proteomics, generating an “adductome” of those proteins in 16HBE cells adducted by ozone derived oxysterol Seco A. NLRP2 was identified as adducted at lysine residue K1019 and the sole adducted target with potential involvement in O_3 induced inflammatory signaling. We recapitulate NLRP2 adduction in Figure 3.7 by utilizing the click chemistry reaction and immunoprecipitation showing NLRP2 protein is adducted by O_3-derived oxysterols. The C-terminus LRR domain acts as a sensor for DAMPs and PAMPs and initiates the NLR protein conformational change for recruitment of the rest of the inflammasome complex proteins (45,46). Biochemical analyses indicate that SecoA adduction to NLRP2 occurs in the LRR region at lysine K1019 shown in Figure 3.4D. Our data propose that O_3-derived oxysterol adduction at this lysine residue in the LRR domain forces the NLRP2 active leaver into a constitutively operative position even in the absence of a stimulus. By modifying NLRP2's activation stop-gap, the inhibitory regulation is turned off leading to enhanced inflammasome signaling, which was shown by increased caspase-1 activity and inflammasome complex formation after O_3 exposure. We also observe decreased caspase 1 activity and inflammatory signaling overall in cells with knocked down NLRP2 expression, further supporting the notion that NLRP2 plays a key role in O_3-induced inflammasome activation in airway epithelial cells. Lastly, we show that inhibiting the final steps in inflammasome signaling, caspase 1 cleavage and IL-1 receptor availability, has the same impact on *IL-6* and *IL-8* expression as knockdown of NLRP2, providing supportive evidence that NLRP2 and inflammasome activity plays a role in airway epithelial cell responses to O_3.

Aside from NLRP2, future attention should be paid to the breadth of proteins adducted by O_3-derived oxysterol Seco A. Within the 130 distinctive proteins adducted only 14 fell outside of the gene ontology classifications of structural/cytoskeletal, chaperone, and cell cycle (Table 3.1). It is not surprising that the most prominent classifications represented in an unbiased proteomics screen are the most abundant proteins in many cell types. In future studies it is important to consider the impairment oxysterol adduction may have on these proteins. For example, oxysterol adduction to cytoskeletal proteins could alter cell membrane integrity similar to the finding presented in previous studies examining oxidized phospholipid adducts in endothelial barrier dysfunction (47,48).

Overall, the data presented within this Chapter serve to link O_3-derived oxysterol-NLRP2 adduction and O_3 induced inflammation in the human airway, presenting a new paradigm underlying the mechanisms driving the response to O_3 exposure. This finding, along with previous NLR protein inflammation studies provide a novel mechanism by which protein inflammation may be governed in the human body and specifically identifies NLRP2 as critical in airway inflammatory response. Considering the significant abundance of NLRP2 in human airway epithelial cells, further studies fully identifying the role for this NLR protein are needed.

3.6 Figures:

Figure 3.1: O3-derived oxysterol protein adduction
Secosterol A (SecoA) and its aldol condensation form Secosterol B (SecoB) derived from cholesterol ozonization. Secosterols react readily with Lys resides of proteins

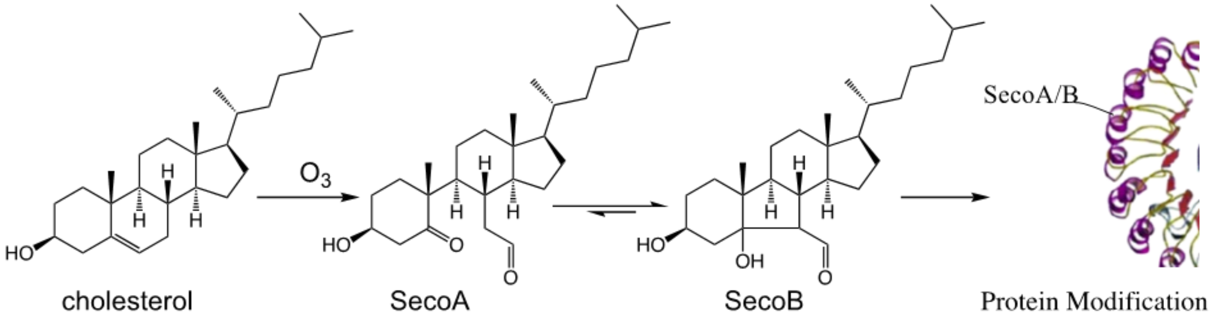

HO
H
H
H
cholesterol
O3
HO
O
H
H
H
O
SecoA
HO
OH
H
H
H
O
SecoB
SecoA/B
Protein Modification

Figure 3.2: Click Chemistry and LC/MS Analysis
The treatment of alkyny-secosterol A (*a*-SecoA) in 16HBE cell line following click reaction to introduce biotin for subsequent immune-affinity purification using Streptavidin beads. The immobilized proteins onto the beads were washed extensively with various solutions (details in the method section) then exposed to hand-held UV lamp (@365 nm) for 2h to release the adducted proteins from the beads. The photo-released proteins were dried in SpeedVac and re-suspended in LDS buffer for short run on SDS-Page LC-MS/MS analysis. (A) *a*-SecoA adducted proteins extended with biotin were probed with IRDye® 800CW Streptavidin. (B) Short stacking SDS-Page gel indicates no signs of proteins in the control (DMSO) lane whereas wholesome proteins of adduction with *a*-SecoA treated cells.

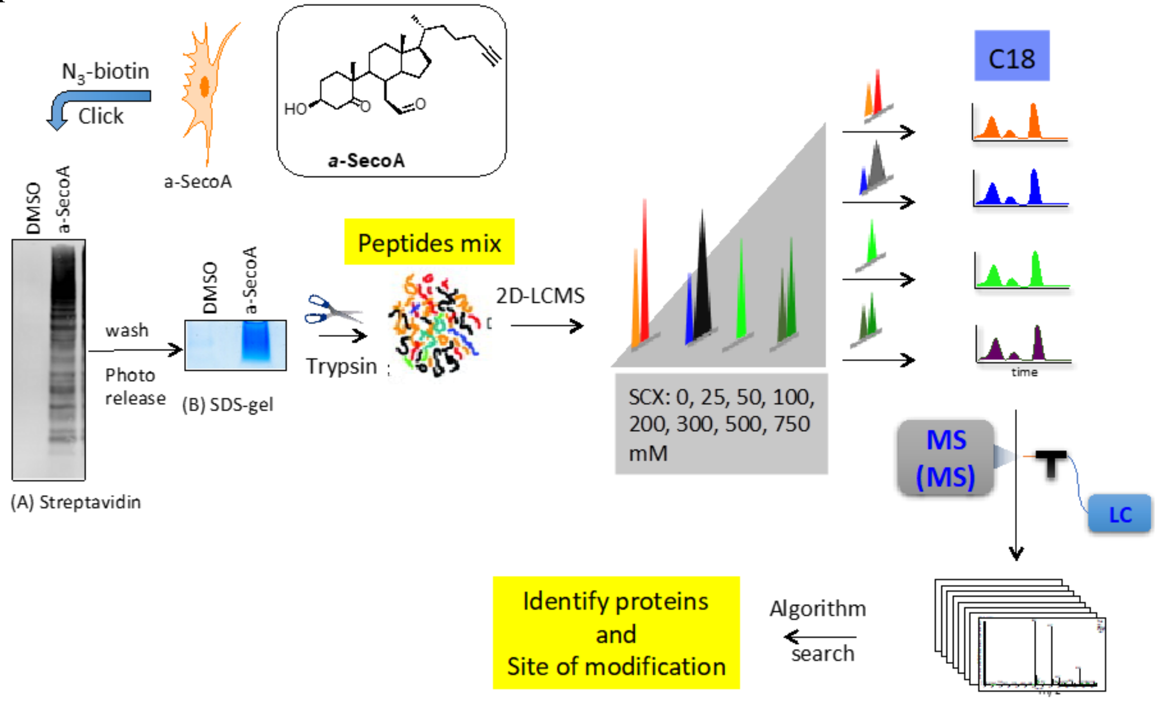
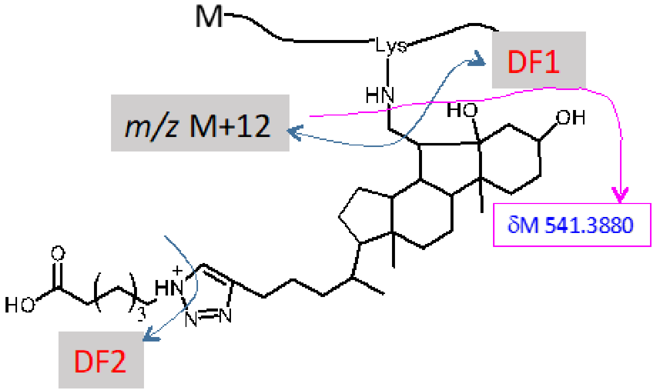

N_3-biotin
Click
a-SecoA
HO
O
O
a-SecoA
DMSO
a-SecoA
wash
Photo
release
DMSO
a-SecoA
(B) SDS-gel
(A) Streptavidin
Peptides mix
Trypsin
2D-LCMS
SCX: 0, 25, 50, 100, 200, 300, 500, 750 mM
C18
time
MS (MS)
LC
Algorithm search
Identify proteins and Site of modification

Figure 3.3: Structure of an *a*-SecoA adduct on a protein lysine after photo-release
These adducts cause a mass shift of 541.3880 of peptide fragments. Diagnostic fragments formed (DF1 and DF2) at m/z 512 and 129 are found in MS/MS of peptide adducts.

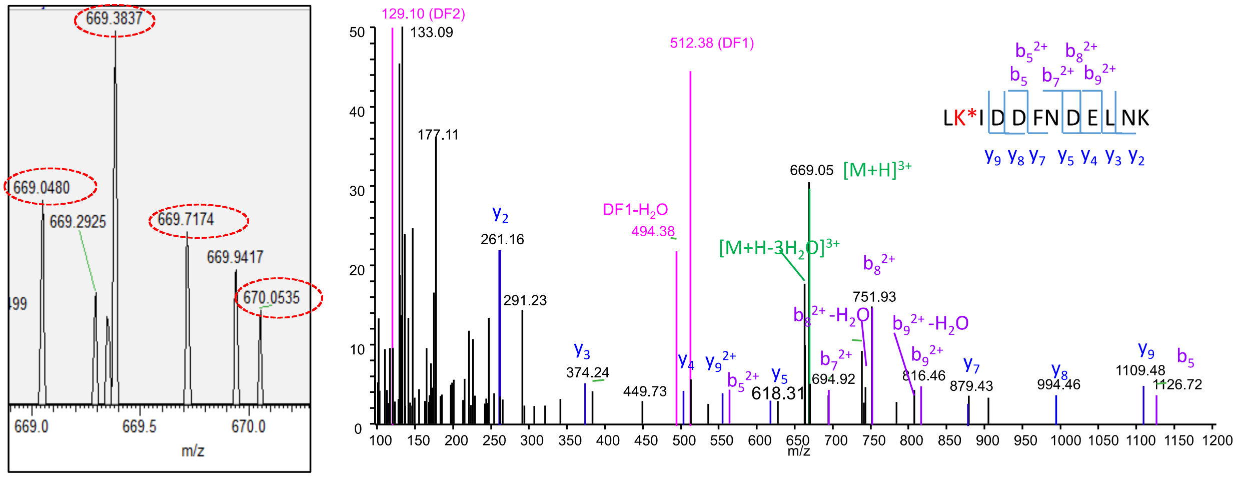

M
Lys
DF1
HN
m/z M+12
HO
OH
δM 541.3880
O
HO
3
+
HN
N=N
DF2

M = peptide

DF1 : *m/z* 512 (-H_2O), DF2 : m/z 129

Figure 3.4: NLRP2 (NACHT, LRR and PYD domains-containing Protein 2) modification with *a*-SecoA

(A) Precursor found with an error of 1.1 ppm, (B) MS/MS of the modified peptide and their b and y ions assignment including DF1 and DF2. (C) 57 distinctive peptides were also found with a total of 218 spectral count. One Lys at the site of 1019 in LRR is modified by *a*-SecoA. (D) NLRP2 structure modeled using cryo-EM structure of NAIP5 from mouse (Mus musculus) with 74% coverage and 100% confidence with Phyre2.



(A) Precursor found *m/z* 669.0480 (δ 1.1 ppm)

(B) MS/MS of the modified peptide, LK*IDDFNDELNK bearing the modification

(C) Total number of spectral counts and peptides belong to NLRP2 (120 kD)

Distinct peptides	Distinct match	Filtered spectra	Modified peptide (reside)	Protein coverage (%)
57	95	218	1 (K1019)	54

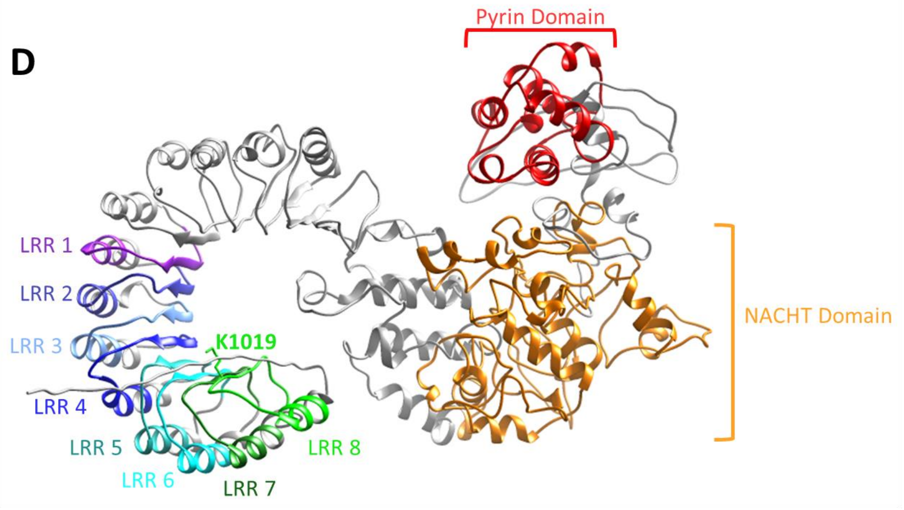


Figure 3.5: SecoA Adductome Protein Association Network

130 SecoA adducted proteins were evaluated by gene ontology and protein association network through string-dB. NLRP2 is identified as a unique functional protein adducted and is reported independent of the association network.

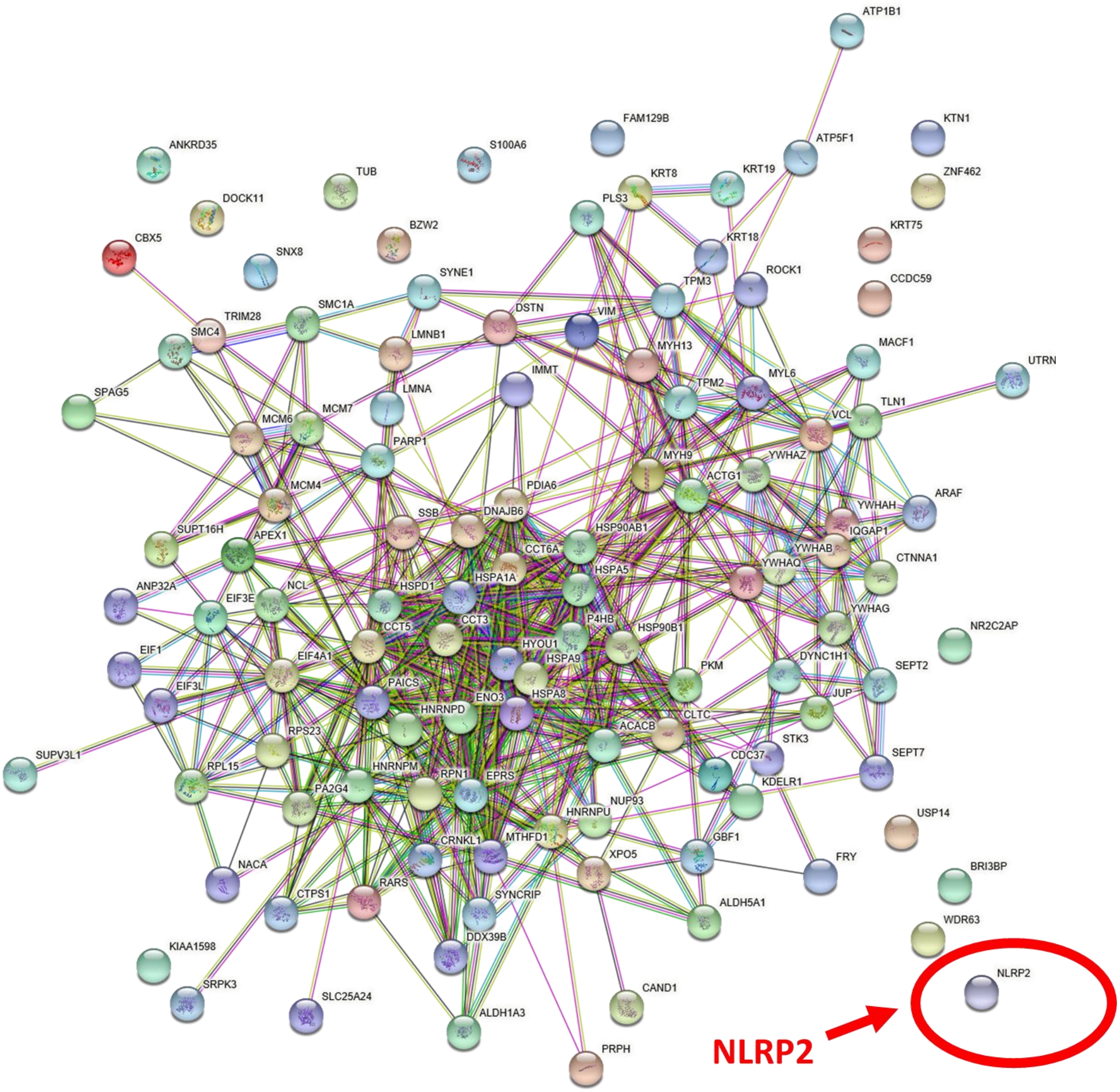

ATP1B1
FAM129B
KTN1
ANKRD35
S100A6
ATP5F1
TUB
KRT8
KRT19
ZNF462
DOCK11
PLS3
CBX5
BZW2
KRT75
KRT18
SNX8
SYNE1
ROCK1
CCDC59
TPM3
SMC1A
DSTN
VIM
TRIM28
SMC4
LMNB1
MYH13
MACF1
IMMT
UTRN
SPAG5
LMNA
TPM2
MYL6
MCM7
TLN1
MCM6
VCL
PARP1
MYH9
YWHAZ
MCM4
PDIA6
ACTG1
ARAF
SSB
DNAJB6
YWHAH
SUPT16H
HSP90AB1
IQGAP1
APEX1
CCT6A
YWHAB
CTNNA1
HSPA1A
YWHAQ
ANP32A
NCL
HSPD1
HSPA5
EIF3E
YWHAG
P4HB
NR2C2AP
CCT5
CCT3
HSP90B1
EIF1
HYOU1
EIF4A1
HSPA9
DYNC1H1
EIF3L
PKM
SEPT2
PAICS
HSPA8
JUP
HNRNPD
ENO3
ACACB
CLTC
RPS23
STK3
SUPV3L1
CDC37
SEPT7
RPL15
HNRNPM
KDELR1
RPN1
EPRS
PA2G4
NUP93
USP14
HNRNPU
MTHFD1
GBF1
CRNKL1
XPO5
FRY
NACA
BRI3BP
RARS
CTPS1
SYNCRIP
ALDH5A1
WDR63
DDX39B
KIAA1598
NLRP2
SRPK3
CAND1
SLC25A24
ALDH1A3
PRPH
NLRP2

Table 3.1: Distinctive SecoA adducted 16HBE proteins

Gene	**Full name**
ACACB	Acetyl-CoA carboxylase beta
ALDH1A3	Aldehyde Dehydrogenase 1 family, member A3
ALDH5A1	Aldehyde Dehydrogenase 5 family, member A1
ATP1B1	ATPase Na/K transporting beta 1 polypeptide
ATP5F1	ATP synthase H+ transporting, mitochondrial Fo comples, subunit B
CTPS1	CPT synthase 1
NLRP2	NLR family, pyrin domain containing 2
SLC25A24	Solute carrier family 25 member 24
APEX1	APEX nuclease 1
ARAF	A-Raf proto-oncogene, serine/threonine kinase
CCDC59	Coiled-coil domain containing 59
NR2C2AP	Nuclear receptor 2C2-associated protein
PARP1	Poly (ADP-ribose) polymerase 1
SRPK3	SRSF protein kinase 3

Figure 3.6: Relative abundance of NLR family protein and mRNA in human airway epithelial cells
Various primary and immortalized human airway epithelial cells, along with other reference cells types, were harvested for total RNA and whole cell lysate for evaluation via qPCR and immunoblot analysis of NLR family proteins. Cells were exposed to filtered air or 0.4 ppm ozone for 4 h and samples were collected 1 h post-exposure. Gene expression levels of NLRP1, NLRP2, NLRP3, NLRP7, and NLRP12 were evaluated in (A) 16HBE, (B) primary HBEC, (C) A549, (D) B2B, (E) THP-1, and (F) K562 cells. Additionally, protein levels for NLRP1, NLRP2, and NLRP3 were evaluated in all the aforementioned cell type by (G) immunoblot. Data are presented as mean ± SEM. Statistical analysis was performed with a one-way ANOVA and Fisher's LSD post hoc test comparing observed means against the respective NLRP2 levels, *p<0.05, n=2.

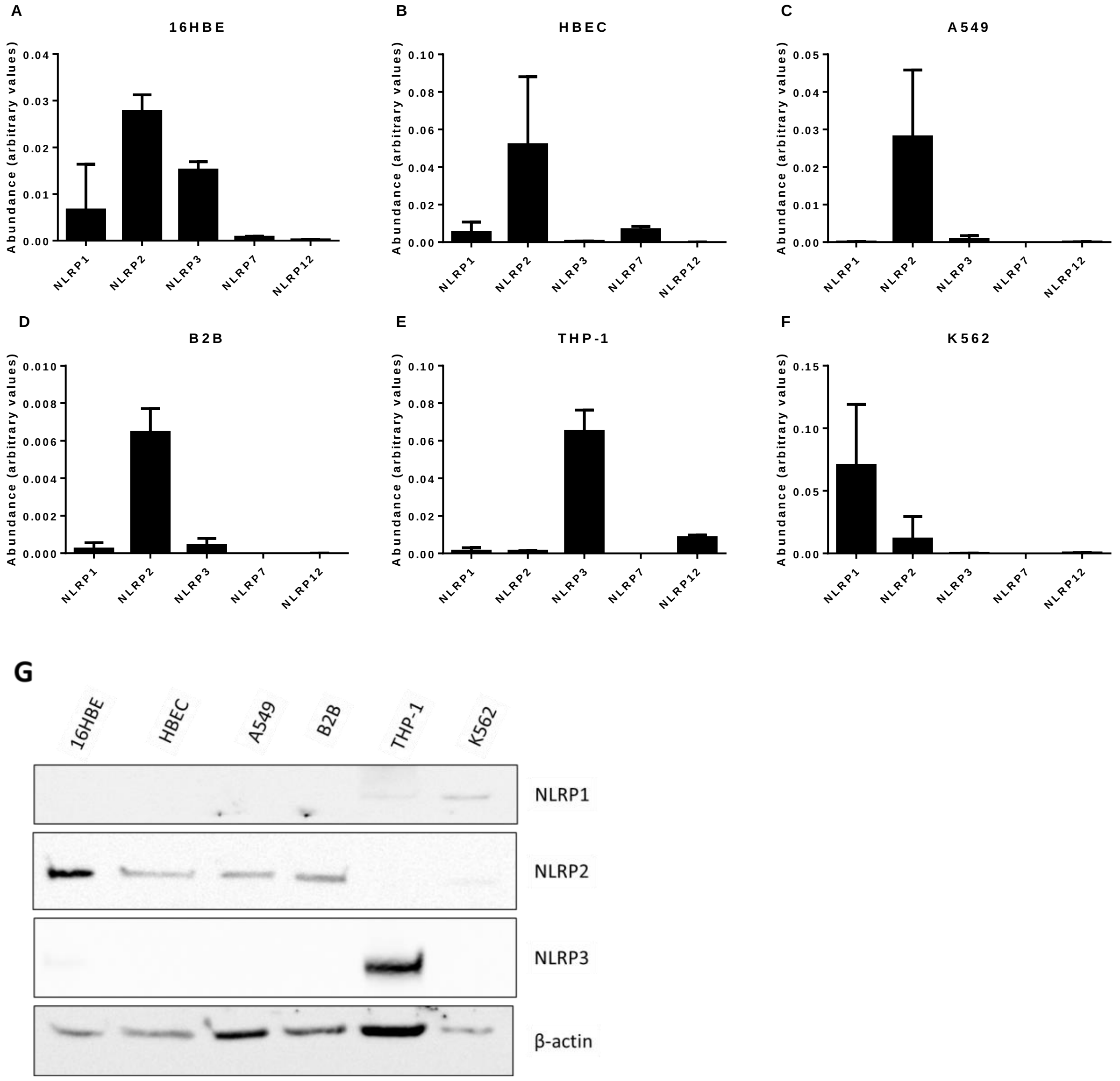


Figure 3.7: Protein catch and photo-release using a-Seco A and a-cholesterol in 16 HBE and THP-1 cells

(A) 20 µM a-SecoA was added in 16HBE cells and THP-1 cells for 4 h and harvested for click and photo-elution. Click mixture was loaded in INPUT serve as control loadings. No signs of NLRP2/3 and HIS3 adductions in DMSO lanes for both cell lines is shown after photo-elution. Only a-SA treated cells were adducted, captured by streptavidin, and photo-released. Abundant levels of NLRP2 is present but not NLRP3 in 16HBE cell line (INPUT) whereas significant levels of NLRP3 but not NLRP2 in THP-1 cell line (INPUT). Interestingly, no NLRP3 adduction is observed in THP-1 cells even if the cells were treated with 20 µM a-SecoA indicating SecoA adduction is highly specific to the protein environment. (B) 16HBE cells were grown in the presence of 20 µM a-Chol prior to ozone exposure. This is true representation of endogenous oxysterols generation and their concentrations when exposed to ozone. Proteins were adducted by alkynylated oxysterols, captured, and photo-released (ELUTED). (C-E) Recapitulated findings utilizing fluorescent probes on additional samples NLRP2 (anti-mouse) is conjugated with IRDye800CW (green) and NLRP3 (anti-rabbit) is conjugated with AlexaFluor680 (red). Indeed the NLRP2 and HIS3 are the targets of the endogenous oxysterols, suggestively alkynyl-secosterols. There is no sign of NLRP3 adduction in 16HBE after ozone exposure.

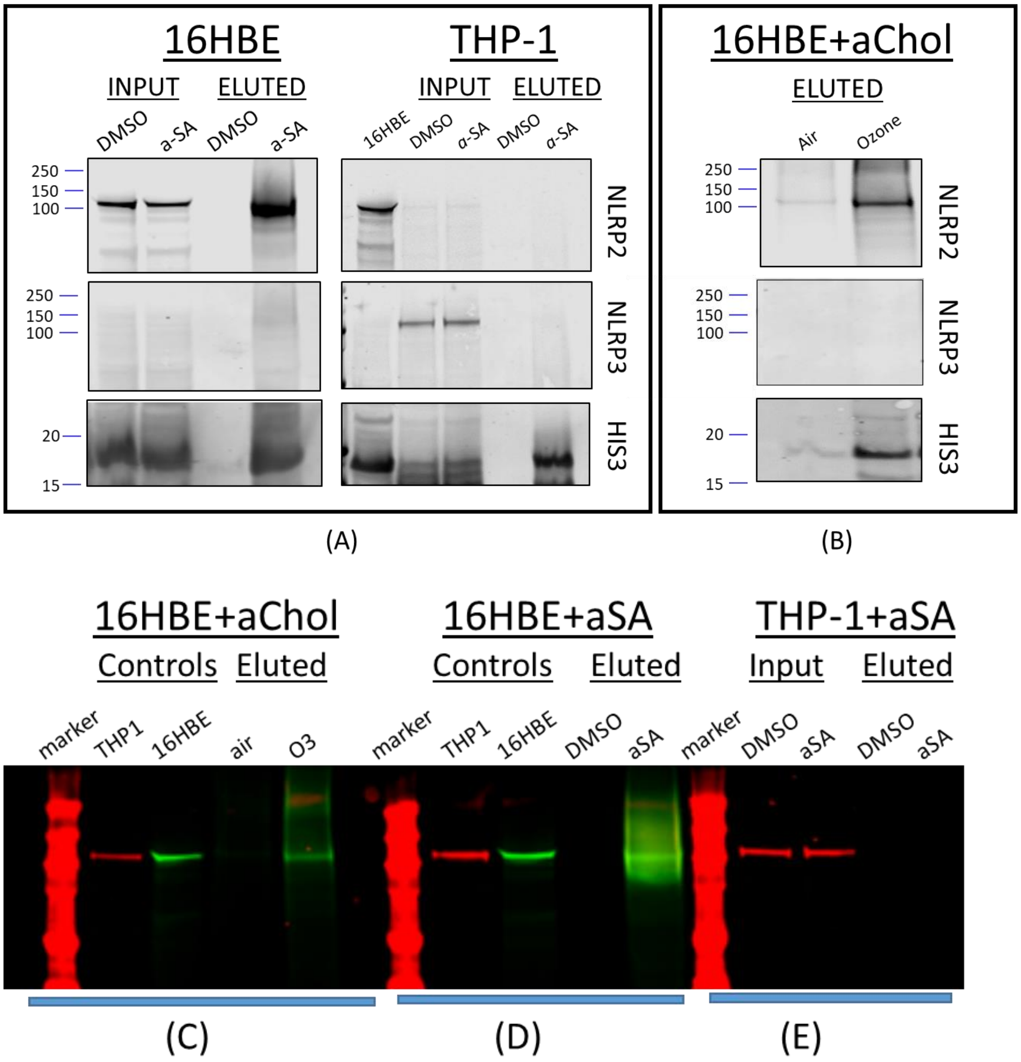

16HBE
THP-1
INPUT
ELUTED
INPUT
ELUTED
DMSO
a-SA
DMSO
a-SA
16HBE
DMSO
α-SA
DMSO
α-SA
250
150
100
NLRP2
250
150
100
NLRP3
20
15
HIS3
16HBE+aChol
ELUTED
Air
Ozone
250
150
100
NLRP2
250
150
100
NLRP3
20
15
HIS3
(A)
(B)
16HBE+aChol
Controls
Eluted
16HBE+aSA
Controls
Eluted
THP-1+aSA
Input
Eluted
marker
THP1
16HBE
air
O3
marker
THP1
16HBE
DMSO
aSA
marker
DMSO
aSA
DMSO
aSA
(C)
(D)
(E)

Figure 3.8: Immunohistochemistry and immunoblot analysis of O_3 induced levels of NLRP2 and inflammasome activity in human airway epithelial cells
A) Representative blot of NLRP2 and Caspase-1 and its cleavage products in whole cell lysate collected 1 h post-exposure from 16HBE cells exposed to filtered air or 0.4ppm ozone for 4h. B) Relative densitometry of NLRP2 from three separate immunoblot experiments. Data are presented as mean ± SEM. Statistical analysis was performed with a one-way ANOVA and Fisher's LSD post hoc test comparing observed means between filtered air and 0.4ppm O_3 $^*p<0.05$, n=3. C) HBECs exposed to air/ O_3 were immunohistochemically stained for DNA (blue), NLPR2 (green) and ASC (red).

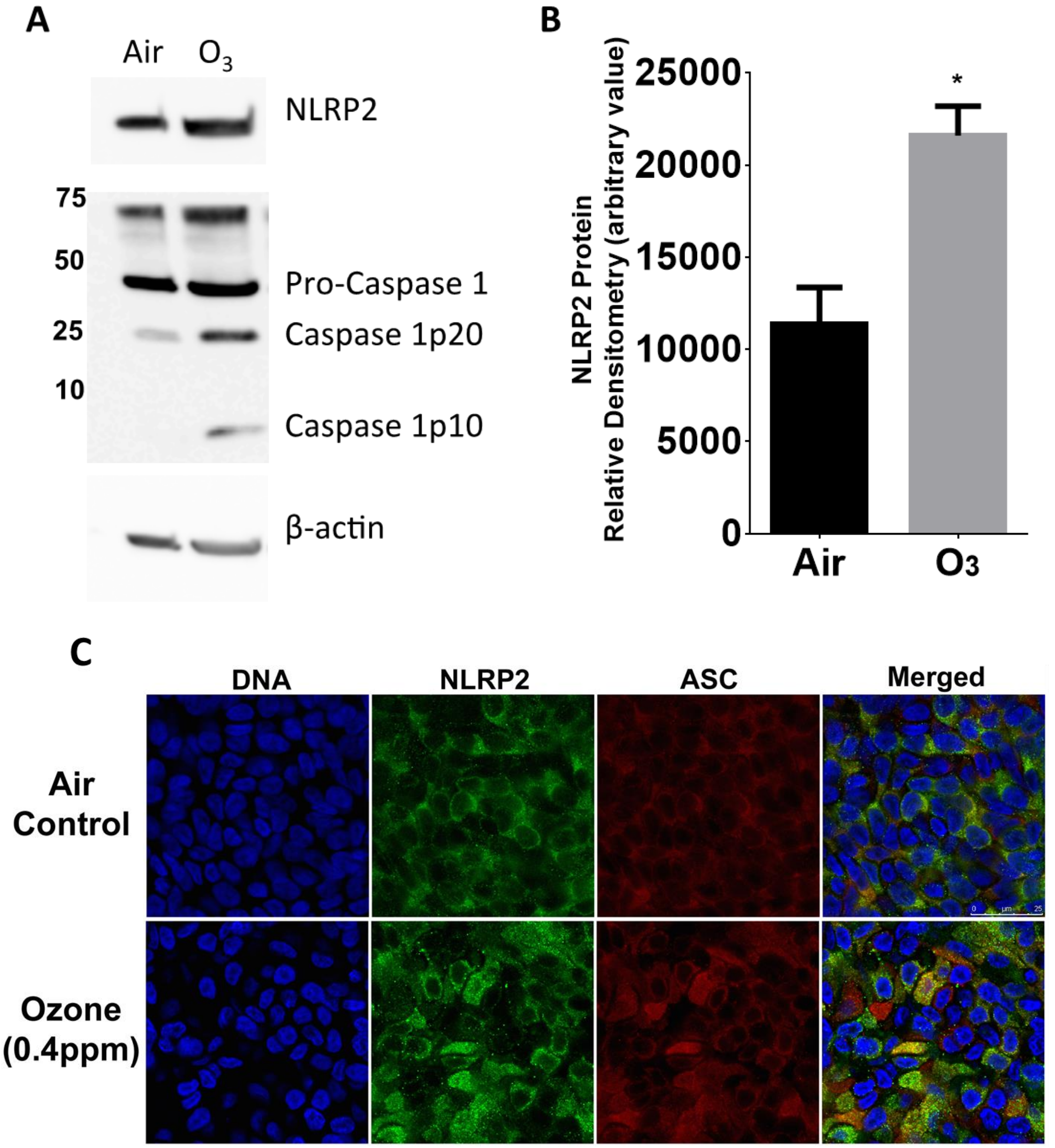


Figure 3.9: shRNA knockdown of NLRP2 in 16HBE cells
A) qPCR measurements of NLRP2 knockdown in 16HBE cells. B) Immunoblot confirmation of shNLRP2 knockdown in 16HBE cells, a representative blot. Data are presented as mean ± SEM. Statistical analysis was performed with a one-way ANOVA and Fisher's LSD post hoc test comparing observed means between filtered air and 0.4ppm O_3 *$p<0.05$, n=3.

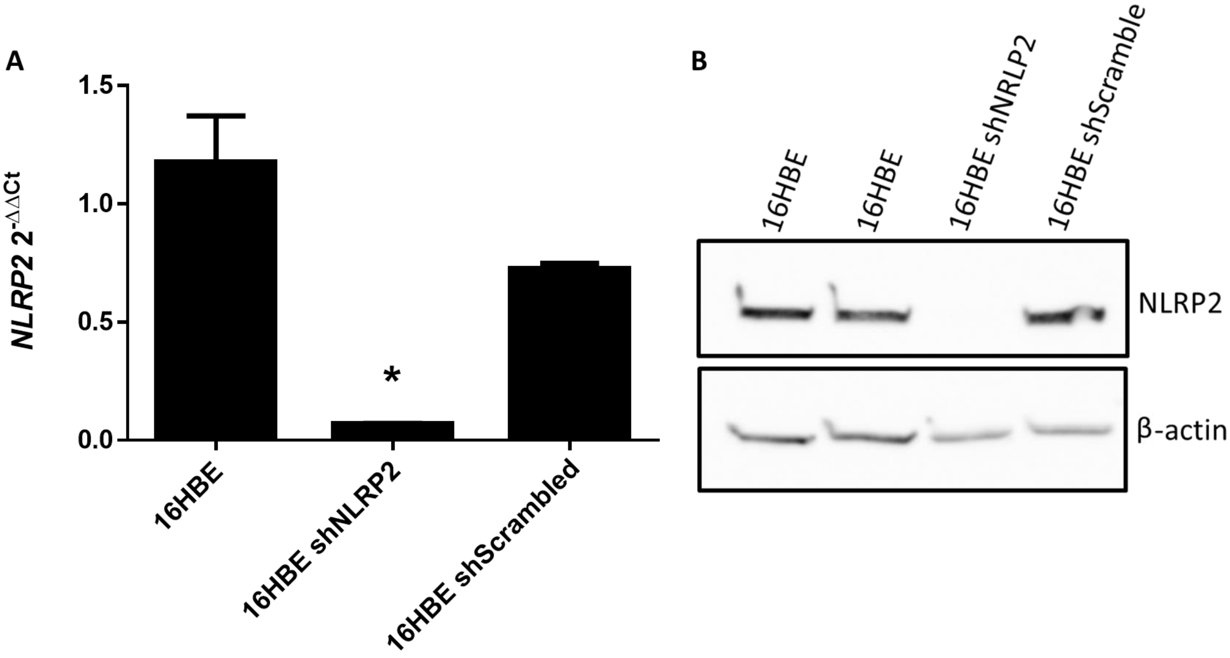


Figure 3.10: NLRP2 shRNA inhibits O_3-induced inflammatory signaling
A) *IL6* and B) *IL8* gene expression in control and shNLRP2 16HBE cells exposed to 0.4ppm O_3 for 4 h and harvested 1 h post. Data are presented as mean ± SEM of fold change compared to the DMSO control. Statistical analysis was performed with a one way ANOVA and Fisher's LSD post hoc comparison test, *$p<0.05$ n=3

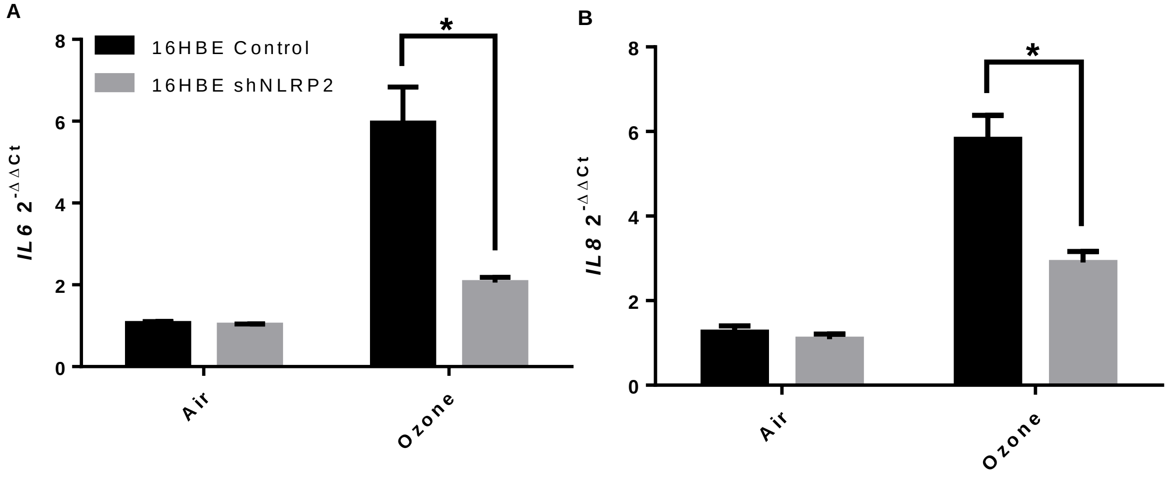
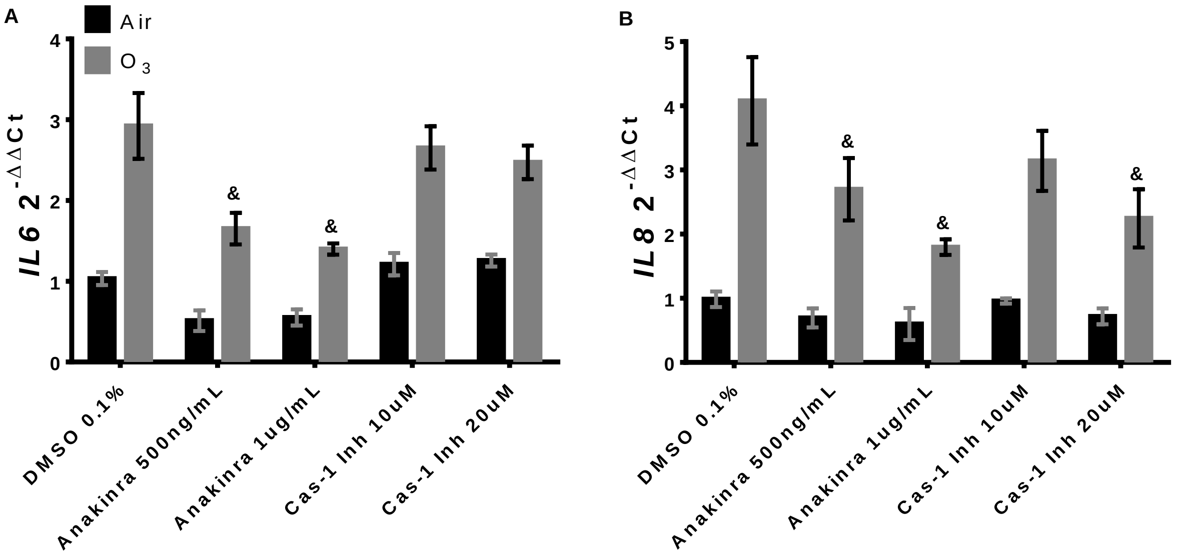


Figure 3.11: Anakinra and Cas-1 inhibitors also inhibit inflammatory signaling in 16HBE cells.
A) *IL6* and B) *IL8* gene expression in 16HBE cells treated with Anakinra at 500ng/ml or 1μg/ml and Caspase-1 inhibitor at 10μM and 20μM for 24 h. The cells were then exposed to filtered air or 0.4ppm O_3 for 4h and total RNA collected 1 h post-exposure. Data are presented as mean ± SEM of fold change "&" indicates comparison to the DMSO control. Statistical analysis was performed with a one way ANOVA and Fisher's LSD post hoc comparison test, *p<0.05 n=3



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CHAPTER 4: THE SMALL MOLECULE ANTIPSYCHOTIC ARIPIPRAZOLE POTENTIATES OZONE-INDUCED INFLAMMATION IN AIRWAY EPITHELIUM

4.1 Overview

Inhaled ground level ozone (O_3) has well described adverse health effects, which may be augmented in susceptible populations. While conditions, such as pre-existing respiratory disease, have been identified as factors enhancing susceptibility to O_3-induced health effects, the potential for drug x pollutant interactions in sensitizing populations or further exacerbating underlying conditions has not yet been studied. In the airways, inhaled O_3 is able to interact with the lipid rich airway lining to generate reactive and electrophilic oxysterol species capable of causing cellular dysfunction and inflammation. Therefore, drugs that modify cholesterol synthesis could have a deleterious biological effect in the presence of O_3. Chemical analysis studies have established the capacity for a variety of small molecule antipsychotic drugs, like Aripiprazole (APZ), to elevate circulating 7-dehydrocholesterol (7-DHC) levels by competitively inhibiting 7-dehydrocholesterol reductase, the enzyme regulating the final step of cholesterol biosynthesis. 7-DHC serves as a highly reactive target for lipid peroxidation and increased oxidative stress. Our results show that APZ, administered at clinically relevant concentrations, and the known DHCR7 inhibitor, AY9944, increase 7-DHC levels and potentiate O_3-induced pro-inflammatory cytokine expression and alter overall gene expression in airway epithelial cells. Additionally, we find that ozonized 7-DHC enhances *IL-6* and *IL-8* expression to a greater extent than ozonized cholesterol. Overall, the evidence we provide describes a potential mechanistic basis for a drug x pollutant interaction between APZ and O_3, posing significant adverse public health implications considering the prevalence of O_3 exposure and APZ use.

4.2 Introduction

According to the World Health Organization, total global air pollution increased 8% between 2011 and 2016, with more than 80% of people living in developed urban settings exceeding WHO air quality guidelines (1). Concurrently, the development of small-molecule pharmaceutical drugs, including antidepressants, has increased exponentially over the last 20 years. In the United States, 59% of the population is taking one or more pharmaceutical drugs and of those people, antidepressant use doubled from 6.8% to 13% between 1999-2012 (2,3). Combined, the pervasive issue of global air pollution and the steady increase of pharmaceutical drug use provides an ever-growing opportunity for biochemical interaction and potential for adverse health effects. Despite the capacity for drugs to biochemically alter the targets for pollutants and potentially exacerbate injury, little research has been conducted on drug x pollutant interactions and human health.

While there are limited well-described drug x environment interactions, such as antibiotic-induced UV photosensitivity by Tetracycline (4-6), even fewer studies have examined this concept in the context of air pollution. Previous studies have demonstrated that in mice, ozone (O_3) exposure potentiates liver injury caused by supratherapeutic acetaminophen doses. Similarly, acetaminophen exacerbates environmental tobacco smoke-induced oxidative stress and sensory irritant-reflex responses in rodent models (7,8). However, the inflammatory potential of other drug x pollutant interactions in the human lung remains completely unknown. This is particularly important for ambient pollutants, such as O_3, as currently more than a third of the

U.S. population lives in areas exceeding the 2015 National Ambient Air Quality Standard for O_3 (0.07ppm for 8-hour average), which are exposure levels known to decrease lung function and cause inflammatory responses in humans (9-11).

O_3 is a highly reactive oxidant gas, capable of damaging organic molecules in human tissue. More specifically, O_3 reacts with pulmonary surfactant and epithelial cell membranes to produce lipid peroxides and oxidant species (12). In the airway, O_3-induced oxidation of cholesterol is known to lead to the formation of electrophilic oxysterols, capable of forming protein adducts, potentially perturbing normal cellular signaling, and increasing inflammation (13-15)). Therefore, drugs that alter cholesterol synthesis could potentially modulate O_3-induced inflammation in the lung. Indeed, epidemiological data indicates that cholesterol-modifying drugs, such as statins, mitigate oxidant stress and air pollutant-induced adverse health effects, yet, little clinical evidence has been presented specifically for O_3 (16,17). More importantly, limited research has examined the potential for other cholesterol-modifying drugs to exacerbate O_3-induced health effects (18,19).

In particular, one of the final steps in the synthesis of cholesterol involves the reduction of 7-dehydrocholesterol (7-DHC) to cholesterol by the enzyme 7-dehydrocholesterol reductase (DHCR7) as illustrated in Figure 4.1A. 7-DHC contains two double bonds on the primary carbon ring (Fig 4.1B/C), making it highly susceptible to O_3-induced oxidation and lipid peroxidation chain propagation (20). Considering the high ozonization potential of this sterol, any cell or fluid with elevated levels of 7-DHC could be sensitized to O_3-induced oxidative stress (21). Therefore, the effects of drugs which modify cholesterol synthesis by elevating the 7-DHC/Cholesterol ratio may increase potential susceptibility to O_3-induced oxidative stress need to be further investigated.

In 2014, the small molecule antipsychotic drug Abilify®, common name: aripiprazole (APZ), often prescribed for off-label use as an anti-depressant or for anxiety, accounted for 8 billion dollars in sales and was ranked 2nd in the U.S. for top selling prescription drugs (22). Notably, APZ inhibits DHCR7 activity in an off-target effect, thereby modifying cholesterol metabolism and leading to accumulation of 7-DHC (23,24). However, whether drugs, such as APZ, known to modify cholesterol synthesis and lead to the accumulation of reactive cholesterol precursors, could potentially exacerbate O_3-induced inflammation, presents a clinically important knowledge gap. Thus far, the potential for drug x pollutant interactions to sensitize populations to ground level O_3 has not yet been studied (25). The increased prevalence of O_3 air pollution and use of prescription small molecule drugs, such as APZ, further illuminates the scope and capacity for drug x pollutant interaction leading to adverse health outcomes. Our study is designed to elucidate the impact of the cholesterol-modifying drug APZ on O_3-induced inflammation in the human airway. We hypothesize that APZ inhibition of DHCR7 increases pulmonary levels of 7-DHC, leading to the formation of highly reactive oxysterol species and potentiation of O_3-induced inflammation.

4.3 Materials and Methods

Cell Culture – 16HBE14o (16HBE) cells, a SV-40 transformed human bronchial epithelial cell line were a gift from Dr. D. C. Gruenert (University of California San Francisco, San Francisco, CA). For experiments performed at air-liquid interface (ALI), 16HBE cells were plated on fibronectin-coated (LHC Basal Medium [Life Technologies, Carlsbad, CA], 0.01% Bovine serum albumin (BSA) [Sigma-Aldrich, St. Louis, MO], 1% Vitricol [Advanced Bio Matrix, San Diego, CA], and 1% human fibronectin [BD Biosciences, San Jose, CA]) 0.4µm

Transwell® plates (Costar, Corning, NY), and grown submerged in minimal essential media (MEM) (Gibco, Thermo Fisher, Gaithersburg, MD) with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin-streptomycin (Gibco), and 1% L-glutamine (Gibco) until confluent for 6 days. For experiments performed with submerged cultures, 16HBEs were plated on fibronectin coated 12 or 24 well plates and given 10% FBS in MEM. For both types of *in vitro* experiments, the serum concentration was reduced to 2% FBS, apical media was removed (if at ALI), and the cells were grown for an additional day before treatment and subsequent challenges.

Pharmaceutical drug treatment of 16HBE cells – APZ (Sigma-Aldrich) and AY9944 (Sigma-Aldrich) were reconstituted in DMSO to create a 10mM stock solution. Submerged cells were given 1mL and 500µL of 1µM APZ treatment for 12 and 24 well plates, respectively. Drug treatments were replenished daily and immediately before O_3 exposure. Treatment of cells with AY9944 was completed in a similar fashion at concentrations of 0.5µM and 1µM.

TNFα agonist treatment – Submerged 16HBE cells were grown in 24 well plates until confluent and given 500µL of 1µM of APZ in 2% FBS MEM. TNFα was reconstituted in DPBS (Gibco) to create a 10µg/mL stock solution; serial dilutions were performed in 2% FBS MEM to make a 20ng/mL solution. After 3 days of pre-treatment with APZ, cells were stimulated with TNFα for 4hrs. Immediately afterwards, RNA was collected for qPCR analysis.

Ozonization of sterols in solution – Compressed Gas, Oxidizing, N.O.S. (oxygen, Nitrous Oxide) (UN3156, Airgas USA, Radnor, PA) was sent through an ozone generator (AquaZone, Red Sea, Huston, TX) and bubbled through Hanks' balanced salt solution with calcium and magnesium (HBSS++) (Life Technologies) containing either 20µM cholesterol (Sigma-Aldrich), 7-DHC (Sigma-Aldrich), or DMSO for 30mins. Solutions were immediately applied to submerged 16HBE cultures for 1hr. Solutions not sent through the compressed gas and ozone generator were also applied directly to cells as a control. Afterwards, RNA lysates were collected and analyzed for *IL*-6 and *IL-8* expression.

In Vitro O_3 Exposure – Cultured 16HBE cells at ALI were exposed to either filtered air or 0.4ppm O_3 for 4hrs in exposure chambers operated by the U.S. EPA in order to mimic the 8hr average exposure of an individual. This dose of O_3 is shown to have minimal cytotoxicity and maximal innate immune response in our 16HBE cells (26). 1hr post exposure, basolateral media was collected, apical sides of the transwells were washed with 110µL of Hanks' balanced salt solution (HBSS) (Life Technologies). RNA was collected in lysis buffer provided by the Pure Link RNA Mini Kit (Life Technologies, Carlsbad, CA) prepared with 1% 2-Mercaptoethanol (Sigma-Aldrich).

Real-time qPCR – Total RNA was isolated from the 16HBE lysates using the RNA kit listed above. cDNA preparation and real-time qPCR were performed as previously described (27,28). The β-actin primer was purchased from Applied Biosystems, Foster City, CA. Human IL-8: 5'-FAM-CCTTGGCAAAACTGCACCTTCAC-TAMRA-3' (probe), 5'-TTGGCAGCCTTCCTGATTTC-3' (sense), and 5'-TATGCACTGACATCTAAGTTCTTTAGCA-3' (antisense) and IL-6: 5'-FAM-CCAGCATCAGTCCCAAGAAGGCAACT-TAMRA-3' (probe), 5'-TATGAAGTTCCTCTCTGCAAGAGA-3' (sense), and 5'-TAGGGAAGGCCGTGGTT-3' (antisense) were prepared in-house. Expression was determined by the ΔΔCt method using β-actin for normalization. ΔCt values were computed by subtracting the threshold cycle (Ct) values for β-actin from the Ct values for the gene of interest. The ΔCt for the control was then subtracted from each sample's value to determine the ΔΔCt values. The fold change in gene expression was then calculated as $2^{-\Delta\Delta Ct}$.

NanoString Gene Expression Analysis – 16HBE mRNA was isolated from samples obtained from cells exposed to 1µM ApZ for 3 days followed by 4 h O_3 exposure and analyzed for gene expression via Nanostring™ (Seattle, WA) nCounter® PanCancer Immune Profiling Panel. Nanostring™ gene expression data were normalized to the geometric mean of stable housekeeping genes and to positive and negative control genes, and analyzed using the nSolver™ software provided by the manufacturer. The heat map was generated using Average Pearson Correlation and unbiased clustering.

Statistical Analysis – All *in vitro* data were performed in at least 3 separate experiments, each with multiple technical replicates. Data shown are mean ± SEM. See figure legends for further information on the specific statistical analysis used for each experiment.

LC-MS/MS 7-DHC measurement – Samples were chromatographed by RP-HPLC using a UPLC BEH C18 column (1.7µm, 2.1mm × 100mm) in Waters Acquity UPLC system equipped with an autosampler (Waters, Milford, MA) and either ESI or APCI in positive ion mode. For ESI, the sterols were separated by 95% 2mM NH_4OAc in MeOH (solvent B) in an isocratic method with a flow rate of 200µL/min, and the mobile phase solvents consisted of 2mM NH_4OAc (solvent A) in water and solvent B. The injection volume was 10µL using a partial loop with needle overfill mode. MS detections were done using a TSQ Quantum Ultra tandem mass spectrometer (ThermoFisher, Waltham, MA), and data was acquired and analyzed using a Thermo Xcalibur™ 2.2 software package.

4.4 Results

APZ and AY9944 increase 7-DHC levels in human airway epithelial cells – Our experimental paradigm treated 16HBE cells with increasing concentrations of APZ or the known

DHCR7 antagonist AY9944 for 1 or 3 days. No morphological changes were observed at any concentration, and our 1µM APZ treatment regimen showed no overt cytotoxicity after 1, 3, or 6d of treatment (Fig. 4.2A). We also quantified the level of 7-DHC in 16HBE cells treated with APZ. Although the 1 day treatments of APZ increased the 7-DHC/Cholesterol ratio, the 3 day 1µM APZ treatment yielded the greatest significant increase in 7-DHC/Cholesterol to a near 1:1 ratio compared to a neglidgable ratio at baseline (Fig. 4.2B). The known DHCR7 antagonist AY9944 also altered sterol levels in an almost identical manner, significantly increasing the 7-DHC/cholesterol ratio to 1 (Fig. 4.2B). Notably, the 7-DHC to cholesterol ratio in both treatment groups was decreased in cells exposed to O_3, perhaps indicating the conversion of 7-DHC into 7-DHC-derived oxysterols.

APZ treatment causes O_3-specific increases in pro-inflammatory cytokine levels – As shown in previous studies, exposure to O_3 causes an inflammatory response in 16HBE cells marked by significant increases in the gene expression of pro-inflammatory cytokines *IL-6* and *IL-8* (Fig. 4.3A-F). APZ treatment with 1µM for 1, 3, and 6 days prior to O_3 exposure significantly potentiated *IL-6* and *IL-8* expression 1.5 to 2 times higher when compared to vehicle (0.01% DMSO) treated cells exposed to O_3 (Fig. 4.3A/B). Protein concentrations of these cytokines followed the same trend, but did not yield significant differences between the APZ and DMSO treated cells in the O_3 exposure group (Fig. 4.3C/D). Treatment with the DHCR7 inhibitor AY9944 similarly increased pro-inflammatory *IL6* and *IL8* levels in O_3 exposed 16HBE cells (Fig. 4.3E/F). Notably, treatment with APZ for any duration did not significantly increase pro-inflammatory cytokine levels when exposed to filtered air. Moreover, the APZ-induced potentiation appears to be O_3 specific as 16HBE cells challenged with another NF-κB agonist, TNFα, did not exhibit the same APZ-exacerbated expression of *IL-6* and *IL-8* (Fig. 4.4A-B).

Nanostring gene expression analysis of APZ and O_3 – Using the NanoString nCounter® PanCancer Immune Profiling Panel, 16HBEs treated for 3 days with 1µM of APZ or DMSO and exposed to air or O_3 were examined for 730 genes, of which 409 were detectable at baseline in our DMSO/Air exposed cells. O_3 exposure alone revealed 222 genes with significantly altered expression. Within the O_3 exposed cells, APZ treatment showed 45 genes with altered expression when compared to the DMSO treated cells (Fig. 4.5A). Of these 45 genes, 18 were upregulated (Table 4.1) and 27 were downregulated (Table 4.2) compared to APZ pre-treatment. Two-thirds of the upregulated genes are involved in defense response and/or regulation of defense response (String-db GO:0006952 and String-db GO:0031347). Additionally, of the 18 upregulated genes, 14 were identified in their involvement in cellular response to stimulus (GO:0051716) (Fig. 4.5B). Functions varied within the 27 downregulated genes, as 9 of the 27 downregulated were associated with macrophage and lymphocyte recruitment, maturation, and inflammation resolution (Fig 4.5C).

Ozonized 7-DHC increases expression of inflammatory cytokines – O_3 was bubbled through HBSS++ buffer with vehicle DMSO, or with 20µM cholesterol, or 7-DHC for 30 minutes. Buffer solutions were immediately added to submerged 16HBE cultures for 1 h, which were subsequently analyzed for *IL-6* and *IL-8* expression. In comparison to the DMSO control, cells treated with buffer containing ozonized cholesterol modestly elevated *IL-8* expression and significantly elevated *IL-6* expression. However, buffer containing ozonized 7-DHC significantly increased *IL-6* and *IL-8* expression to a greater extent in comparison to ozonized DMSO (Fig. 4.6A-B). Remarkably, there was also a moderately convincing increase in *IL-8* expression in ozonized 7-DHC compared to ozonized cholesterol ($p \leq 0.075$). No significant changes in *IL-6* or *IL-8* expression were observed between the DMSO and sterol groups not subjected to ozonization.

4.5 Discussion

The increasing prevalence of both pharmaceutical drug use and environmental pollutants in the developed world highlight the need to study their potential interactions and combined effects. In order to fill this knowledge gap, we explored the impact that drugs with DHCR7 inhibitory effects may have on O_3 induced inflammatory response in human airway epithelial cells. We observed that APZ, a known DHCR7 inhibitor, significantly increased O_3-induced pro-inflammatory cytokine expression and elevated the 7-DHC to cholesterol ratio. Further supporting this finding, ozonization of buffer containing 7-DHC significantly enhanced the ability to increase the expression of *IL-6* and *IL-8* to a greater extent than ozonized cholesterol did (Figure 4.6). Taken together, these data provide a mechanistic basis for a drug x pollutant interaction and drug-induced augmentation of O_3-induced adverse health effects.

As we describe above, APZ and similar small molecule drugs commonly prescribed for depression are widely used in the US with nearly 10 million prescriptions filled annually (22,29). While APZ is designated an antipsychotic, only 2.5% of the U.S. population are diagnosed as such and it is commonly prescribed in combination with other drugs to treat depression, tic disorders, and irritability associated with autism (30). Circulating levels can reach up to 0.7µM levels in patients with severe bipolar and schizophrenic disorder, informing our use of 1µM as physiologically relevant (31,32). Our data demonstrate that APZ treatment intensifies the well-described O_3-induced inflammation in human airway epithelial cells (33,34). In Figure 4.3, APZ treatment regimens at 1µM over 3 days significantly increased O_3-induced inflammatory cytokine expression almost 2-fold over the vehicle treated cell response to O_3. We did not observe a significant change in IL-6 or IL-8 at the protein level in Figure 4.3C and D. This discrepancy is likely due to the timing of collection and further studies over longer time points are necessary. Our broad gene expression array data suggest that, in addition to increased *IL-6* and *IL-8*, APZ treatment diminished O_3-induced expression of *MIF*, *S100A12*, *MICA*, and *IFNAR1*, all genes associated with immune defense response when compared to the DMSO control treatment. In total, APZ treatment altered O_3 response expression in 44 genes listed in tables 4.1 and 4.2. These data suggest that individuals taking Abilify® on high air pollution days could be susceptible to increased inflammatory responses or adverse conditions in the airway. More broadly, pharmaceuticals with unknown modes of action or side effects could lead to interaction with environmental exposures and unintended adverse outcomes.

It should be noted that APZ treatment may have impacts on the airway aside from potentiating O_3-induced inflammation. The Nanostring array data shown in Figure 4.5 show that APZ treatment has the potential to alter O_3-induced 16HBE gene expression, which may have an independent impact on airway health. For example, the highest observed fold change increase in gene expression following APZ treatment compared to DMSO was in the integrin subunit alpha 1 (*ITGA1*) by 1.4 fold. An increase in ITGA1, which is known to be associated with cell-cell adhesion and inflammatory signaling and could also contribute to the sterile neutrophil influx and immune cell dysfunction associated with O_3 exposure (35). Additionally, the array data and protein association networks in Figure 4.5C show that cells pre-treated with APZ prior to O_3 exposure experienced significant decreases in the expression of various defense innate immune responses. Notably, *MAPK11*, *TP53*, and *HRAS* are all down regulated. Alterations in the expression of these genes could result in serious dysfunction of the important cell proliferation and survival MAPK/ERK pathway. Although we see an increase in *IL-6* and *IL-8* transcription after APZ treatment, our gene expression panel and association network in Figure 4.5B/C also reveals a significant decrease in inflammatory mediator expression. Notably, *MYD88* expression, an IL-1 receptor signaling pathway protein that leads to NF-κB activation and enhanced IL-8 formation is downregulated following APZ treatment. Conversely, NF-κB2 and TNF are reported as significantly up regulated in APZ alone treated cells. Ultimately, the APZ-x-O_3 interaction leads to a varied gene expression response and may serve to magnify the adverse health outcomes associated with inflammatory response and host defense response.

Although the airways are not a significant contributor to the production of endogenous cholesterol, our previous studies show that exposure to O_3 significantly increases the levels of electrophilic oxysterols formed in the healthy human airway compared to individuals exposed to only filtered air (13). 7-DHC represents a unique target for ozonization as the additional double bond at C7 makes the molecule oxidatively unstable, allowing the molecule to be highly reactive towards free radical oxidation and one of the best chain-carrying molecules studied to date (36-38). Any alteration in cholesterol homeostasis and an increase in available 7-DHC could lead to the creation of highly reactive oxysterol species. The drug APZ is a known inhibitor of DHCR7, and increases the 7-DHC/cholesterol ratio in various human cell types (15,39), including airway epithelial cells, as shown in data presented here (Figure 4.2B). The oxidized lipids formed due to 7-DHC ozonizaton are different than those formed due to cholesterol ozonization and have the potential to be more electrophilic and more rapidly react with available proteins and cellular components. The electrophilic oxysterol species structure determines its reactivity with cellular components, thus, it is important to consider any drug-induced modulation to available sterol content in the airway and the potential to generate oxysterol species. We concede that isolation and measurement of 7-DHC ozonization products in human airway epithelial cells treated with

APZ would benefit our model greatly. However, the product mixture from 7-DHC ozonization is extremely complex and stable primary products have not yet been unambiguously assigned (Dr. Ned Porter; personal communication), which is a limitation of this study.

Due to this limitation, we aimed to link the potential for APZ to inhibit DHCR7 and increase the ratio of 7-DHC/cholesterol in airway epithelial cells as the primary mechanism for the potentiated O_3 effect. Although we cannot present direct evidence of DHCR7 inhibition by APZ in 16HBE cells, it has been shown previously in Neuro2A cells (24,39), and we show here that APZ significantly increases levels of 7-DHC in human airway epithelial (Figure 4.2B). Additionally, use of the commercially available DHCR7 inhibitor AY9944 provided a tool to examine the effects of DHCR7 inhibition on O_3-induced inflammation. Similar to APZ, AY9944 increased the 7-DHC/cholesterol ratio and potentiated O_3 induced inflammatory effects in 16HBE cells. The similar effects of AY9944 and APZ on 7-DHC/cholesterol ratio, support our hypothesis that APZ inhibition of DHCR7 and increased abundance of 7-DHC mediates the potentiation of O_3 induced inflammation.

In order to link 7-DHC ozonization products with enhanced inflammatory gene expression without the ability to directly measure the reactive byproducts, we conducted experiments directly stimulating cells with ozonized sterols derived from either 7-DHC or cholesterol. As shown in Figure 4.6, our data demonstrate that the ozonized 7-DHC products significantly enhance *IL-6* and *IL-8* expression more than ozonized cholesterol products. We also show the ability of APZ to enhance markers of inflammation is specific to O_3 exposure. Specifically, *IL-6* and *IL-8* expression in16HBE cells exposed to TNF-α were not affected by APZ (Fig 4.4), indicating that the effects of APZ-induced enhancement of *IL-6* and *IL-8* expression are not generally applicable to all pro-inflammatory stimuli, but to specific to oxidants, such as O_3. Together, these data suggest that the more reactive oxysterol species derived from the ozonization of 7-DHC recapitulated the increased inflammatory responses seen in O_3-exposed 16HBE cells treated with APZ.

Our study highlights the consequence of drug induced cholesterol modification in the presence of oxidant pollutants. Aside from APZ, different classes of therapeutic drugs have the potential for off-target cholesterol modification including other small molecule anti-depressants, and β-blockers. Our investigative team is in the process of extensively examining the potential for various pharmaceutical drugs to inhibit steps in cholesterol synthesis, enhancing the formation of 7-DHC (24). Table 4.3 highlights a few drugs that are among both the highest potency for DHCR7 inhibition in Neuro2A cells and frequency prescribed in the U.S. population. This analysis indicates that the effects of drugs modifying DHCR7 activity and O_3-induced inflammation are not limited to small molecule antidepressants, such as APZ, but also include other classes of commonly prescribed drugs. Specifically, we propose future studies to look at widely prescribed drugs known to inhibit DHCR7 and their influence on O_3 induced inflammation. These data underscore the need for additional comprehensive screening of commonly used drugs for potential pollutant interaction and induced adverse health effects.

Overall, our research findings support the hypothesis that APZ-induced inhibition of DHCR7 and subsequently elevated 7-DHC levels potentiate O_3-induced inflammation in the human airway. These findings also describe a previously unknown drug x pollutant interaction. While there are a limited number of previously described drug x pollutant interactions, we believe our findings are indicative of a larger number of potential interactions that have yet to be uncovered. With ever increasing use of pharmaceutical prescription and ubiquitous air pollution globally, there is a clear need for a new paradigm in toxicity testing examining drugs with the potential to enhance pollutant-induced health effects.

4.6 Figures

Figure 4.1: APZ inhibition of cholesterol synthesis pathway, 7-DHC and cholesterol structures
(A) Cholesterol synthesis pathway and impact of APZ on DHCR7. (B) Chemical structure of cholesterol and (C) 7-DHC.

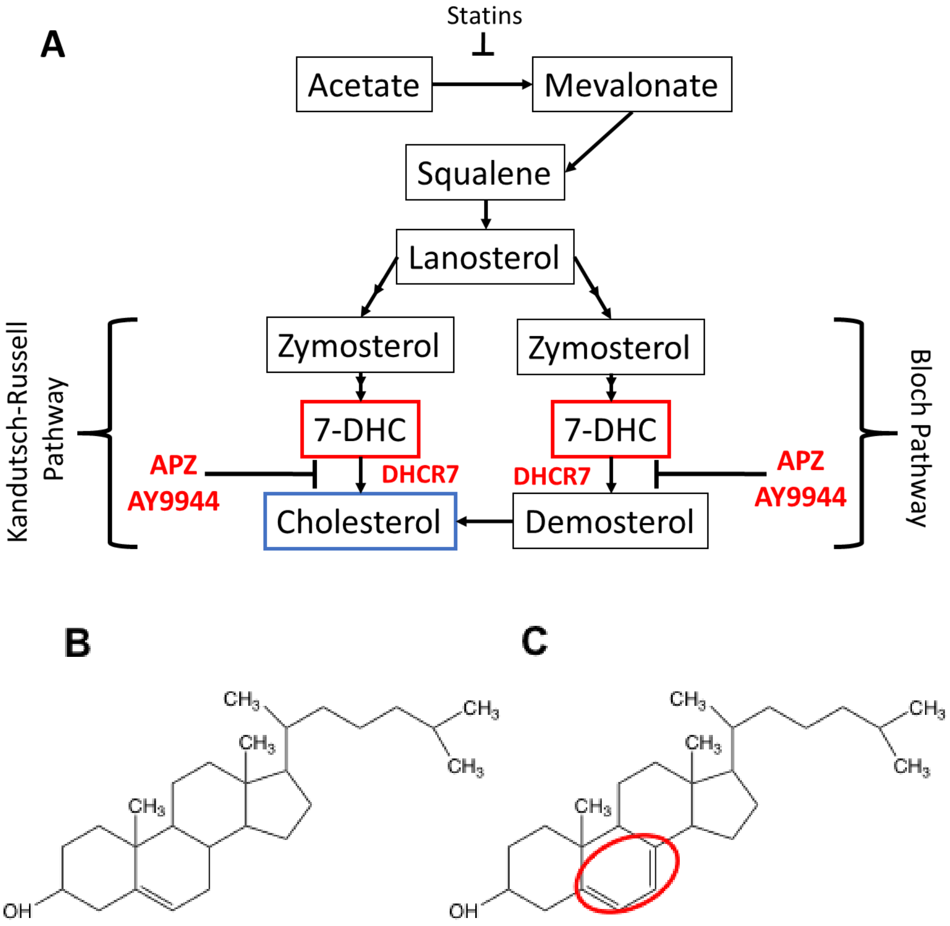
A
Statins
Acetate
Mevalonate
Squalene
Lanosterol
Kandutsch-Russell Pathway
Zymosterol
Zymosterol
Bloch Pathway
7-DHC
7-DHC
APZ
AY9944
DHCR7
DHCR7
APZ
AY9944
Cholesterol
Demosterol
B
C
CH3
CH3
CH3
CH3
CH3
OH
CH3
CH3
CH3
CH3
CH3
OH

Figure 4.2: APZ and AY9944 effects on 7-DHC levels in airway epithelial cells
(A) LDH release assay demonstrated no overt cytotoxicity at 1µM APZ for 1 and 3. LC-MS/MS analysis revealed an increase in the ratio of 7-DHC to cholesterol in 16HBE cells grown at ALI, given (B) 1µM APZ or (C) AY9944 for 3 days compared to 1 day. n=4-8 for (A) and n=3 for (B). Data are presented as mean ± SEM. Statistical analysis was performed using a one-way ANOVA for (A) and a two-way ANOVA for (B). *p≤0.05, ****p≤0.00001 compared to respective DMSO control treatment.

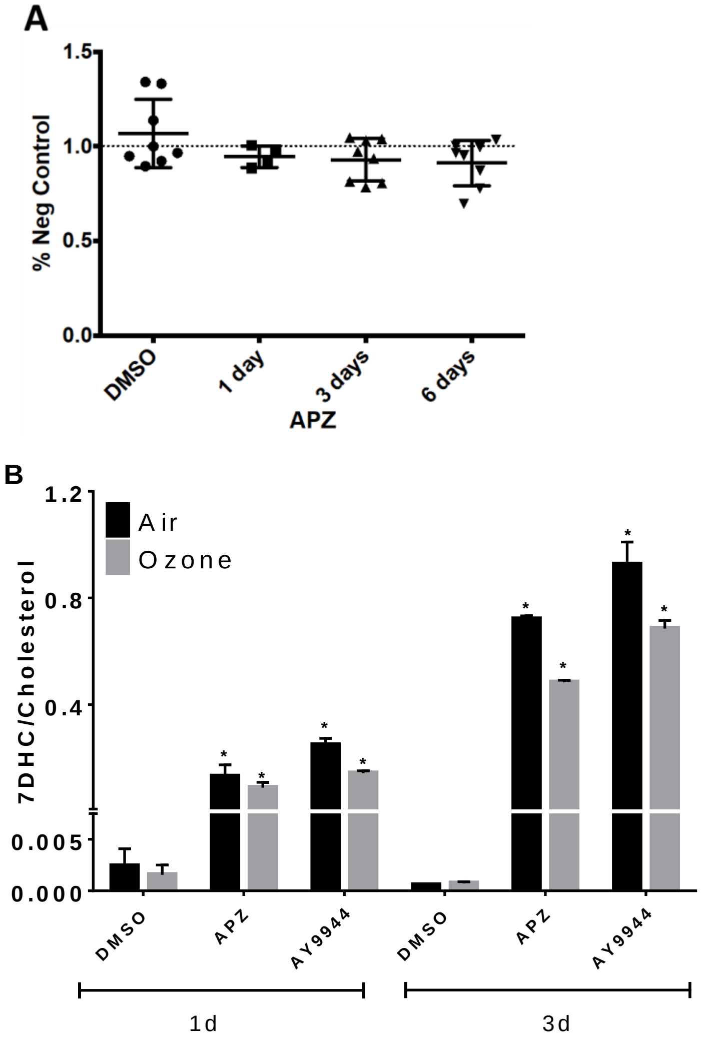

Figure 4.3: APZ potentiates O$_3$-induced *IL-6* and *IL-8* expression
16HBE cells grown at ALI treated basolaterally with 1μM concentration of APZ 1d, 3d, and 6d prior to 0.4ppm O$_3$ exposure yielded enhanced expression of (A) *IL-6* and (B) *IL-8* with significance increases in both markers after 3d APZ treatment. Prolonged APZ treatment prior to O$_3$ exposure also increased (C) IL-6 and (D) IL-8 protein levels. Treatment with 0.5μM and 1μM AY9944 for 3d enhanced (E) *IL-6* and (F) *IL-8* expression. (A-B) n=3-5, (C-E) n=3. Data are presented as mean ± SEM. Statistical analysis was performed using a two-way ANOVA. *p≤0.05, **p≤0.01.

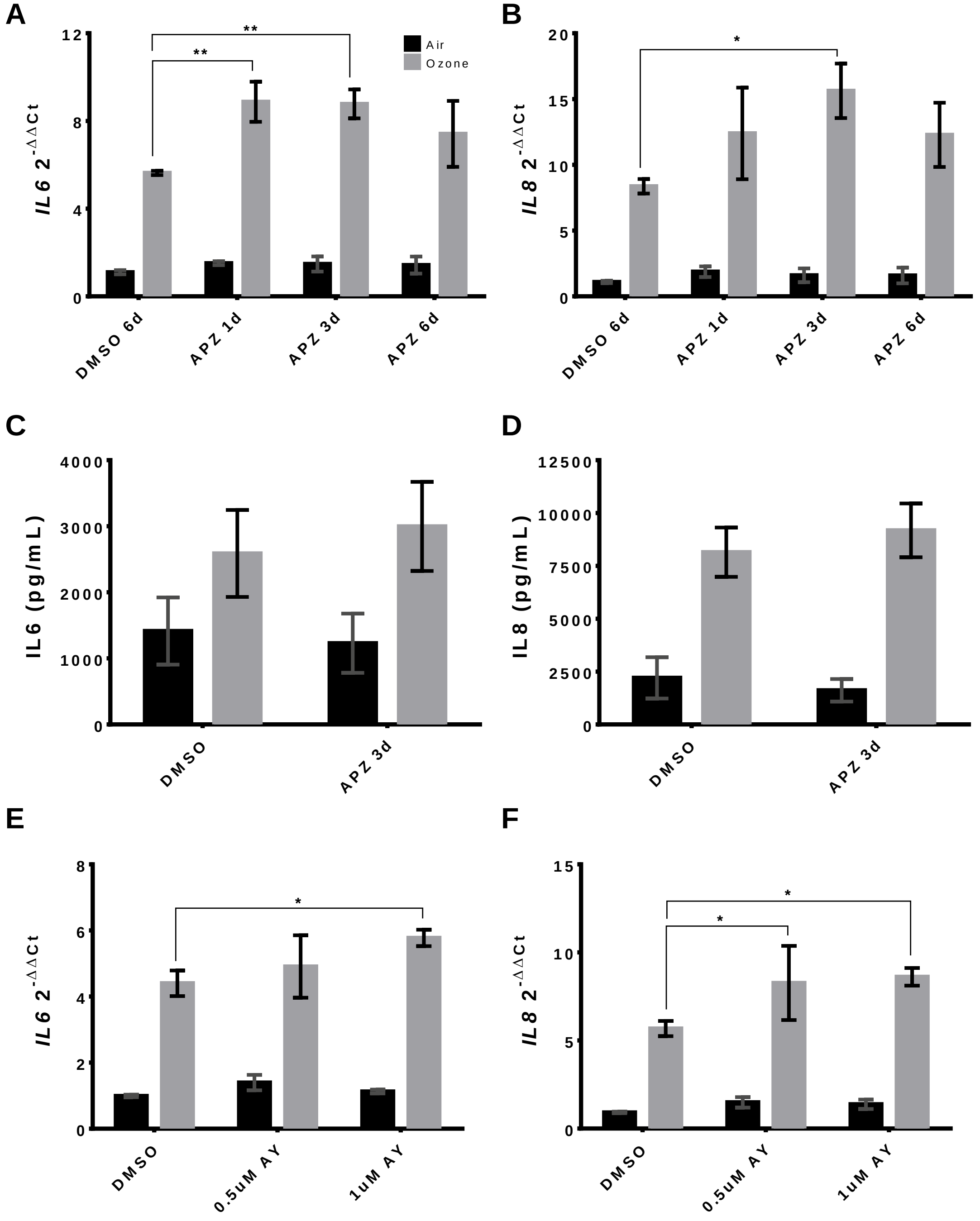

Figure 4.4. Stimulation with TNFα does not incite APZ-induced *IL-6* and *IL-8* expression. Treatment with 1μM APZ or AY9944 for 3d followed by 20ng/mL TNFα treatment for 4h did not potentiate the any change in O_3-induced (A) *IL-6* and (B) *IL-8* expression, n=4. Data are presented as mean ± SEM. Statistical analysis was performed using a two-way ANOVA.

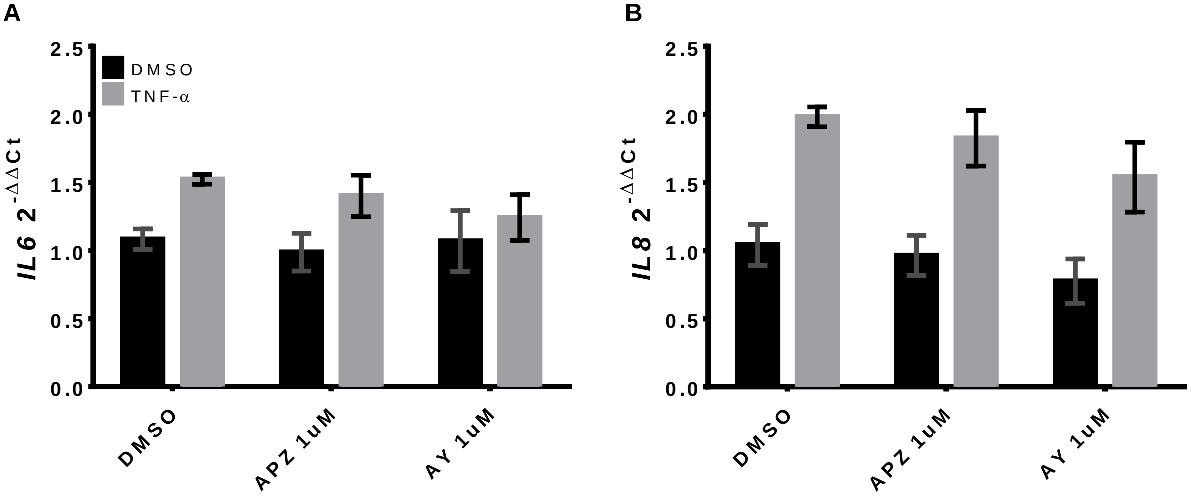

Figure 4.5. Nanostring analysis of gene expression in 16HBE cells treated with APZ
(A) Unbiased heat map of gene expression for cells exposed to O_3. Cells treated with DMSO and cells treated with 1µM APZ for 3d resulted in distinct gene expression profiles. Significantly (B) upregulated and (C) downregulated expression of genes in APZ treated cells exposed to O_3 compared to the O_3 exposed vehicle control. Upregulated genes involved in defense response are shaded red (GO:0006952) and genes involved in the regulation of defense response are shaded blue (GO:0031347) in (B). Down regulated genes involved in cellular response to stimulus are shaded red in (C). n=3. Genes in (A-C) identified using a paired, two tailed t-test comparing O_3 exposed groups ($p \leq 0.05$).

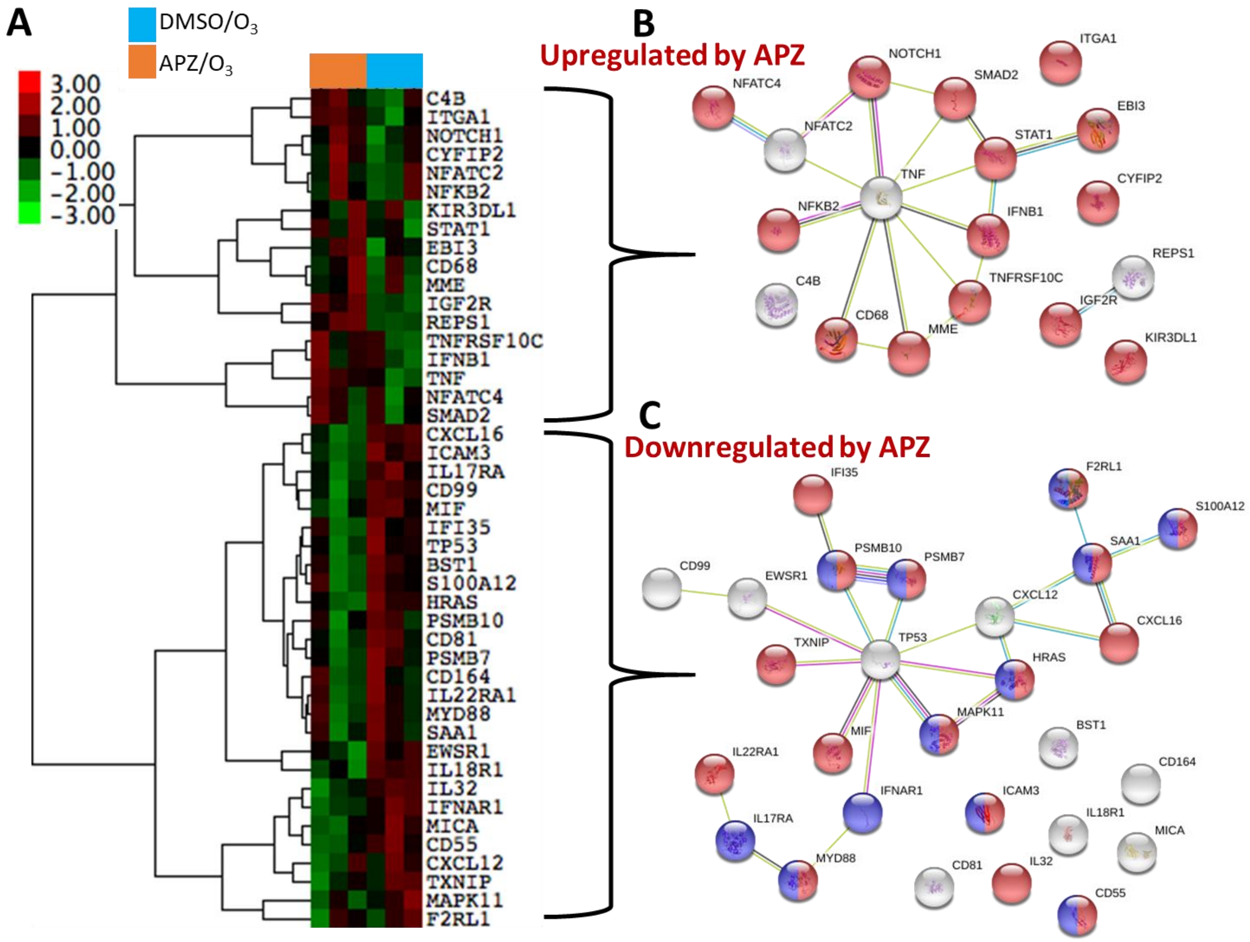

Table 4.1. APZ upregulated genes after O_3 exposure. Significantly upregulated genes organized by p value. Mean ± SEM. Paired two tailed t-test.

Gene	DMSO/O_3 Count	APZ/O_3 Count	p-value
EBI3	52.43 ± 5.51	66.45 ± 5.91	0.00104
STAT1	2722.07 ± 135.98	3002.31 ± 132.24	0.00141
KIR3DL1	34.50 ± 5.09	37.61 ± 4.97	0.00329
IGF2R	689.89 ± 12.07	863.15 ± 27.85	0.00866
SMAD2	1790.46 ± 72.88	1851.71 ± 79.48	0.01201
NOTCH1	343.62 ± 27.12	401.25 ± 22.16	0.01220
NFATC2	922.61 ± 130.38	1025.80 ± 134.32	0.01246
TNF	1563.69 ± 59.08	1755.18 ± 36.17	0.01585
MME	585.77 ± 99.67	701.65 ± 106.51	0.02065
CYFIP2	77.12 ± 3.49	85.24 ± 4.71	0.02214
ITGA1	58.81 ± 6.77	82.14 ± 3.31	0.02265
IFNB1	172.72 ± 24.86	214.98 ± 29.02	0.02592
CD68	182.82 ± 24.42	214.30 ± 28.14	0.02725
NFATC4	45.36 ± 5.04	49.01 ± 4.51	0.02890
NFKB2	2647.99 ± 188.55	2808.19 ± 171.01	0.03151
TNFRSF10C	91.86 ± 10.78	112.77 ± 10.62	0.03499
REPS1	1502.32 ± 3.02	1633.30 ± 25.90	0.04145
C4B	22.33 ± 3.02	28.33 ± 2.63	0.04545

Table 4.2. APZ downregulated genes after O_3 exposure. Significantly down regulated genes organized by p value. Mean ± SEM. Paired two tailed t-test.

Gene	DMSO/O_3 Count	APZ/O_3 Count	p-value
PSMB10	1850.21 ± 129.37	1659.82 ± 136.52	0.00142
HRAS	724.77 ± 22.48	620.90 ± 27.38	0.00324
CD81	6198.99 ± 107.31	5886.74 ± 88.93	0.00400
CD164	8218.67 ± 232.19	8013.24 ± 218.99	0.00427
MIF	15623.29 ± 751.64	12323.69 ± 744.10	0.00744
IFNAR1	562.88 ± 17.11	482.03 ± 10.01	0.00851
F2RL1	2726.62 ± 248.21	2519.25 ± 253.95	0.00863
CD55	75.85 ± 2.14	66.74 ± 1.91	0.01062
MICA	673.65 ± 26.85	581.86 ± 24.91	0.01204
IL22RA1	162.35 ± 15.93	140.07 ± 14.15	0.01371
PSMB7	14021.10 ± 440.69	12979.44 ± 307.28	0.01710
CD99	9202.72 ± 218.41	7421.10 ± 451.48	0.01940
EWSR1	7060.98 ± 147.24	6397.58 ± 240.29	0.01945
IL32	14114.88 ± 192.75	12173.38 ± 468.21	0.01956
BST1	133.37 ± 4.12	118.26 ± 6.08	0.02019
SAA1	13701.20 ± 2461.14	10812.05 ± 2102.12	0.02053
TXNIP	6795.88 ± 583.26	5493.28 ± 508.51	0.02147
IFI35	1320.91 ± 70.83	1134.87 ± 83.84	0.03119
S100A12	147.15 ± 25.30	96.12 ± 34.67	0.03251
CXCL16	3553.24 ± 29.85	3039.91 ± 90.17	0.03269
ICAM3	247.92 ± 17.21	169.93 ± 7.93	0.03402
MAPK11	50.37 ± 4.67	40.08 ± 3.49	0.03485
MYD88	791.43 ± 40.11	733.22 ± 49.12	0.03581
CXCL12	40.69 ± 8.06	28.98 ± 8.48	0.03824
TP53	3425.43 ± 159.45	2979.50 ± 140.66	0.04366
IL18R1	444.04 ± 5.74	363.54 ± 20.97	0.04893
IL17RA	353.29 ± 8.48	318.48 ± 7.94	0.04929

Figure 4.6: Ozonized 7-DHC increases *IL-6* and *IL-8* expression to a greater extent than ozonized cholesterol

20µM 7-DHC, cholesterol and the DMSO vehicle control were ozonized in buffer for 30 minutes and immediately added to submerged 16HBE cultures for 1 hour. The same sterol solutions were also added to submerged cells without ozonization as a control. (A) *IL-6* and (B) *IL-8* expression was determined with qPCR. n=3. Data are presented as mean ± SEM. Statistical analysis was performed using a two-way ANOVA. *p≤0.05, **p≤0.01.

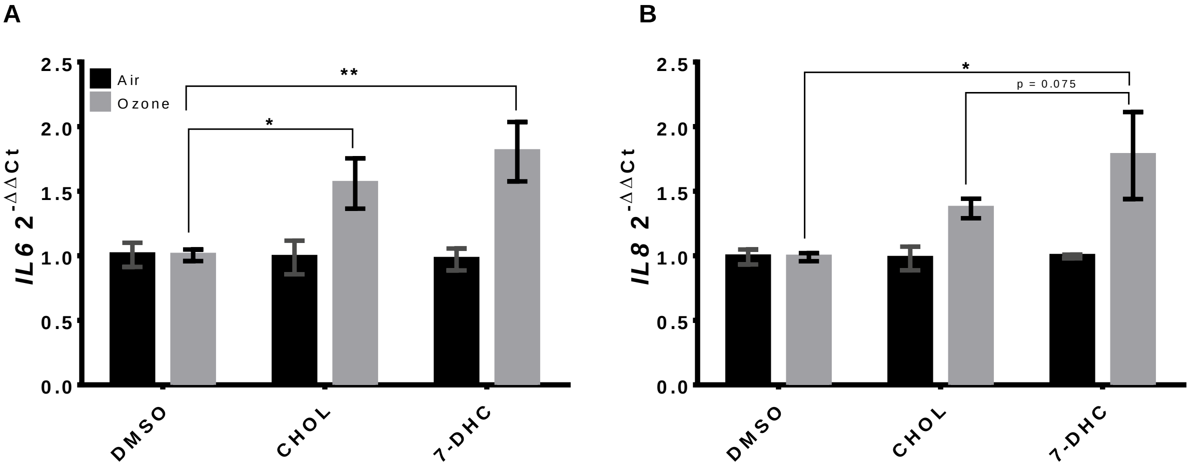

Table 4.3: DHCR7 Inhibiting Pharmaceutical Drugs

Selected pharmaceutical drugs from the 2014 top 200 selling drugs in U.S. Drugs are organized by sales rank and Z-score for DHCR7 inhibitory effect in Neuro2A cells treated with 1μM concentration for 4 h. Adapted from figure by Ned Porter and Hye-Young Kim.

Drug [Common Names]	U.S. Sales Rank 2014	Z-score	Drug Type
Metoprolol [Lopressor, Metolar]	6	63	β-blocker
Trazodone [Desyrel, Oleptro]	28	120	anti-depressant
Buspirone [Buspar]	95	110	anti-anxiety
Oxybutynin [Ditropan, Lyrinel]	107	30	anti-muscarinic
Donepezil [Aricept]	108	80	cholinesterase inhibitor
Aripiprazole [Abilify]	119	200	anti-psychotic
Risperidone [Risperdal]	128	10	anti-psychotic
Nebivolol [Bystolic, Nebilet]	141	234	β-blocker

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CHAPTER 5: IMPACT, NEW KNOWLEDGE GAPS, AND FUTURE STUDIES

The work, both original and referenced in this dissertation, serve to answer pertinent questions regarding the biochemical mechanisms driving O_3-induced inflammation, oxysterol-protein adduction, and the potential for interaction between common pharmaceuticals and environmental pollutants. Despite providing new knowledge in these branches of research, fresh knowledge gaps emerge from our data and original investigations must be proposed to advance our collective knowledge. This chapter will underscore the fields of study that stand to be transformed due to the new data discussed in this manuscript with each section 1) synthesizing our key findings, 2) emergent knowledge gaps, and 3) proposed future studies. Our findings advance current knowledge on O_3-induced inflammatory signaling, O_3 exposure response, airway epithelial inflammasome activity, oxysterol-protein adduction, and drug-x-environment interaction, all of which can be expanded upon in future research endeavors.

5.1 O_3 Disruption of Sterol Homeostasis

5.1.1 Key Findings

As discussed in previous chapters, we must note that any changes in cholesterol synthesis, sterol ratio, and sterol homeostasis can lead to cellular dysfunction and adverse health effects. Dysregulation of oxysterol levels, either by overexpression of CYP enzymes or deletion of synthesis enzymes, impair induction of LXR target cholesterol transport genes and can lead to sterol overload and ultimately cell death (1). Chapter 2 presents compelling evidence that O_3-derived oxysterols adduct to LXR and influence the inflammatory response following O_3 exposure. Specifically, SecoA inhibits the transcription of sterol transport and binding proteins, increasing NF-κB activity and inflammatory cytokine signaling. It is of additional interest, however, that O_3 exposure itself alters expression of genes that regulate cholesterol efflux, fatty acid synthesis, and cholesterol regulation. Our findings indicate that cholesterol availability is paramount to the formation of O_3-derived oxysterols in the airway, suggesting novel targets to modify effects associated with ambient O_3 exposure.

5.1.2 Emergent Knowledge Gaps

Besides LXR-oxysterol adduction and impaired cholesterol transport gene expression we have little evidence regarding the effects of oxysterols on downstream LXR signaling and cellular functions following O_3 exposure. We show that O_3 exposure alone decreases the transcription of cholesterol transport genes regulated by LXR activity in human airway epithelial cells, however, it remains unknown whether the observed decrement in cholesterol transport could be associated with additional adverse health effects. LXR agonists have long been studied as a means to enhance airway immune response and attenuate diet-induced or genetic dyslipidemia (2). Additionally, KO mice deficient in cholesterol transport capabilities (e.g. ABCG1) experience poor innate and adaptive immune response to bacterial and environmental stressors in the lung suggesting cholesterol efflux enhances innate immune function. (3). Clinical reports also draw connections between “good” High Density Lipoprotein (HDL) cholesterol serum levels and positive airway proficiency test results (4,5).

Beyond the airway, it is possible that the cardiovascular events associated with O_3 exposure may also be enhanced by dysregulation of cholesterol homeostasis due to air pollution. Epidemiologic and clinical studies show a correlation between high O_3 pollution and increased cardiovascular events. O_3 exposure can increase vasoconstriction, tachycardia, and increased mortality attributed to cardiopulmonary events (6-8). Further, O_3-derived oxysterols SecoA and SecoB have been measured in atherosclerotic lesions and lipid overloaded macrophages, and could be a causative factor in the atherogenesis (9). Additionally, new evidence exists that air pollutants alter systemic cholesterol in humans leading to cardiovascular disease. Individuals living in areas with high traffic-related air pollution have lower levels of HDL and increased oxidized Low Density Lipoprotien (ox-LDL) a phenotype associated with cardiovascular disease (10,11). LXR activity and cholesterol homeostasis may be a key factor in protecting the airway from pollutant induced inflammatory diseases and certainly requires further study.

5.1.3 Future Studies

Three main avenues of research present themselves in our newly uncovered knowledge gaps, 1) determining the extent to which O_3 exposure alone alters sterol transport and binding mechanisms, 2) further elucidate the mechanism by which O_3 derived oxysterols alter LXR signaling, and 3) analyze the association between respiratory/cardiovascular health and changes in LXR signaling and sterol content that start in the airway. First, cell line and animal knock out models for LXR, ABCA1, SREBP1 exposed to O_3 should be evaluated by LC/MS for altered overall sterol profiles as well as response to the pollutant, revealing whether there is a synergistic effect between diminished cholesterol homeostatic signaling and environmental exposure. Second, the specific consequences of O_3–derived oxysterol-LXR adduction can be elucidated by probing the LXR transcription pathway. For example, LXR activation occurs through

SUMOylation, conjugation and dimerization with RXR before transcribing cholesterol transport genes (12). O_3 or SecoA exposed 16HBE cell lysate could be immunoprecipitated with RXR or SUMOylation antibodies and probed for LXR in order to determine if LXR adduction interferes with these steps leading to transcription. Third, animal model and clinical studies could examine the connections between LXR signaling and O_3-induced adverse health effects. LXR agonists have already been proposed as a therapeutic target to reduce inflammatory disease progression in everything from liver disease, atherosclerosis, and to atopic dermatitis, however the specificity and potency of these treatments vary from tissue to tissue and few have been tested in the context of an environmental exposure (13-16). In the case of the airway, lipid metabolic therapy by LXR inducing agents may be able to suppress the generation of O_3-derived oxysterols, reduce their uptake by pulmonary macrophages, and attenuate the progression of pulmonary diseases and atherogenesis. LXR agonists could be tested as a potential rescue treatment for mouse models of air pollutant induced acute lung injury and atherosclerosis.

5.2 Oxysterols: A Piece of the O_3-Induced Inflammation Puzzle

5.2.1 Key Findings

Ground level O_3 air pollution has long been studied as a potent initiator of inflammation in the human airways, has been associated with various disease states, and exacerbation of existing disease. The mechanisms governing O_3-induced inflammation however, have never been fully elucidated and represent a much studied "black-box" in understanding inflammation in the lung. Many mechanisms play a role in O_3-induced inflammation and the research detailed in this dissertation describes a new piece of the puzzle. First, we now know that O_3-derived oxysterols alone have the capacity to activate NF-κB signaling in 16HBE cells and we believe that oxysterol-protein adduction is tied to this inflammatory response. Second, as discussed in chapter 2, the LXR activation pathway is known to have a transrepressive effect on NF-κB inflammatory activity and oxysterol adduction to LXR may inhibit inflammation repression leading to greater induction of inflammatory cytokine signaling. Finally, we go further in chapter 3 to show that oxysterol-NLRP2 adduction may also play a part in O_3-induced inflammation. Previous studies have already shown that IL-1β and IL-18 signaling induces NF-κB activity and increases levels of IL-6 and IL-8 (17). Our data supports this finding, connecting inflammasome activation with NLRP2 activity in 16HBE cells. Although we cannot confirm the link, NLRP2 inflammasome caspase-1 activity and subsequent cleavage of IL-18 and IL-1β, this process appears to regulate the expression of inflammatory cytokines *IL-8* and *IL-6*. Our data support the notion that oxysterol formation and protein adduction in the human airways contribute to O_3-induced inflammatory signaling pathways, presenting a novel field for additional studies.

5.2.2 Emergent Knowledge Gaps

New questions about the mechanisms behind O_3-induced inflammation arise from our reported data. Foremost, the oxysterol-protein adductome needs to be more thoroughly examined as adduction to any of the reported proteins may change cellular function. The main oxysterol-protein targets discussed in this dissertation were highly focused and may not reveal the extent that O_3-derived oxysterols and protein adduction can impact cellular function. In Chapter 2, LXR was chosen as it is a known target for enzymatically derived oxysterols and in chapter 3 NLRP2 was chosen out of our adductomics analysis based on its unique abundance in 16HBE cells and its proclivity as a sensor for damage associated molecular patterns among the other adducted targets. Thus, it is of interest to determine whether O_3-derived oxysterol adduction of chaperone and cytoskeletal proteins alter cellular viability and health. Aldehydic adduction of chaperone heat shock proteins has been shown to arrest cellular transcription and decrease HeLa cell viability (18) . Second, we must question whether oxysterol formation and adduction occurs specifically with O_3 or could other free radical and oxidant pollutants also create oxysterols in the airway or elsewhere. Finally, the role of NLRP2 activity as a possible regulator of inflammatory signaling in human airway epithelial cells must be further elucidated. NLRP2's abundance in airway epithelial cells may mean it has a prominent function outside of inflammasome assembly. NLRP2 could most likely be a sensor for damage and pathogen associated molecular patterns (DAMPs and PAMPs) aside from O_3. Testing other agonists such as bacterial LPS or TNF-α may reveal if NLRP2's response to O_3 is specific. Finally, it is still unclear whether NLRP2 induced IL-18 and IL-1β presents a positive feedback mechanism inducing inflammatory cytokine transcription. Answering these questions may further describe the mechanisms driving O_3 induced inflammation and illuminate NLRP2's undiscovered role in airway epithelial cells.

5.2.3 Future Studies

Despite presenting compelling data exemplifying novel biochemical mechanisms governing O_3-inuced inflammation, additional pieces of the puzzle need to be filled in by future studies. Crystalline structure analysis of adducted NLRP2 and LXR could provide better evidence of how O_3-derived oxysterols influence normal protein activity. Further, a site directed mutagenesis study on NLRP2 activity could provide more clues regarding its specific mechanism in airway epithelial cells and deletion of the adducted lysine residue adducted prevents adduct induced inflammasome activation.

Finally, our "adductomics" analysis already shows that oxysterol-protein adduction is not limited to NLRP2 and LXR and various other adducted protein targets may also be involved in inflammatory response in the airway. A more in-depth study of the "adductome" including the location of adducts and possible conformation changes to protein structure need to be considered when evaluating the total influence oxysterol-protein adduction has on O_3-induced inflammation in the airway epithelium. Chapter 2 focused on adduction of LXR, which we pursued since it was a known sensor for oxysterols and was indeed adducted by O_3-derived oxysterols. NLRP2 was targeted in the second paper as a standout protein associated with inflammasome signaling adducted by SecoA, however, focusing on the many other adducted proteins was beyond the scope of this dissertation. Scaffolding, Cell cycle and Structural proteins were all highly adducted by SecoA and the potential adverse effects of their adduction needs to be discussed. Previous studies have shown the ability for electrophilic aldehydes like 4-HNE to adduct to cytoskeletal elements and alter cellular viability in various cell types (19,20). Clinically, cytoskeletal modification has been associated with adenocarcenomas, and lipid peroxidation product modification of microtubules may contribute to neuronal dysfunction and Alzheimer's disease (20,21). In our airway epithelial cells, membrane and monolayer integrity could be measured by epithelial resistance and visualized using immunohistochemical staining for cytoskeletal elements in order to measure any deficit in cellular organization.

5.3 Novel Target: NLRP2 in Airway Epithelial Cells

5.3.1 Key Findings

Although the focus of our translational minded research primarily examined the biochemical mechanisms of inflammation and adverse health effects following O_3 exposure, I must stress the importance of our novel finding of NLRP2 as an abundant protein in the human airway epithelium. Finding NLRP2 in our list of adducted proteins was unexpected, as the unbiased proteomics approach produced NLRP2 among mainly large and abundant chaperone, cytoskeletal, and histone proteins. Prior to these reported findings there was no record of NLRP2 in human airway epithelium cells or a clearly described function in any human tissue. For the first time, our research revealed the high abundance of NLRP2 in both primary human airway epithelial cells and widely used human airway epithelial cell lines. Our data serves to clarify elements of its role in inflammasome activation, however, further studies are necessary.

5.3.2 Emergent Knowledge Gap

Despite the distinctive finding of NLRP2 as a key player in airway epithelial cell response to damage associated molecular signal, like oxysterols, there is a significant gap of information regarding its function in the human body that needs to be addressed. Firstly, NLRP2 was found to be highly abundant in human airway epithelial cells (on par with expression levels of β-actin and other highly expressed genes) it may have functions beyond inflammasome activation. We know O_3 exposure can alter NLRP2 function, is it possible that other oxidant gasses and environmental exposures impact NLRP2 function. Second, we confirm that NLRP2 is clearly present in airway epithelial cell types but not in immune cells like monocytes and macrophages. The relative abundance of NLRP2 in different cell types creates an enigma for future studies, as especially in the airway, epithelial cells coexist with resident macrophages, which rely on inflammasome activity for pathogen response (22,23). Finally, NLRP2 may pose a significant target to study outside of the airway and may be a key cellular protein in epithelial cells in other tissues.

5.3.3 Future Studies

Additional research is necessary in order to better describe NLRP2 function in human airway and in the human body in general. Although the knowledge gaps regarding NLRP2 are vast, we can start by examining NLRP2's function as a whole and design tests to ascertain if NLRP2 can be targeted by environmental and pharmaceutical intervention. NLRP2 is highly conserved in primates, however, studies evaluating NLRP2 in mouse models is not consistent in regards to its function (24). NLRP2 knockout mouse models reveal its primary role in embryogenesis but no change in the lung phenotype of the animal (25). Although present in mouse models, murine NLRP2 may not be a direct homologue of the human form. Creating a humanized NLRP2 mouse model would allow us to mimic its role in the human lung inflammasome assembly and better explain the mechanism by which environmental exposures illicit downstream inflammatory signaling in the human airway. In the case of O_3-derived oxysterols, adduction appears to occur more readily with NLRP2 as compared to the more frequently studied NLRP3, meaning NLRP2 may be an undervalued target for adverse cellular stress. Careful characterization of where NLRP2 is expressed in the human body is needed. The Human Protein Atlas provides only uncertain evidence regarding NLRP2, showing low RNA expression of NLRP2 in all tissue types with slightly more in the testes No protein level evidence for NLRP2 is recorded in the Human Protein Atlas. Thus, the cells in tissues frequently affected by oxidative stress should be assessed for NLRP2 presence and function. Endothelial cells in particular would be of interest as they are highly susceptible to oxysterol exposure as measured in atherosclerotic plaques.

5.4 Intrinsic and Extrinsic Factors Determine O_3 Exposure Response

5.4.1 Key Findings

In the field of O_3 exposure research, primarily intrinsic factors such as age, sex and genetics have been considered when evaluating intensity of O_3 response in an individual. Numerous clinical studies show that individuals exposed to O_3 can be classified into two groups, responders and non-responders, based on the reduction in forced exhalation volume (FEV) and neutrophil infiltration following exposure (26,27). The majority of current literature examining responders and non-responders indicate that these two classifications are primarily due to genetic predisposition to O_3 exposure response by activity of the Glutatione-S-Transferase M1 (GSTM1) gene. GSTM1null individuals are thought to have a diminished capacity for resolving O_3 induced inflammation and respond to the exposure more profoundly than those individuals with normal GSTM1 alleles (28-30). Similarly, *in vivo* mouse strain genomics studies reveal genetic mechanisms of susceptibility to O_3 adverse health outcomes including TNF, TLR4 and MHC genes (31-33). While the intrinsic factor of genetics has explained some of the differential susceptibility in human O_3 response, extrinsic stressors such as diet, and pharmaceutical drugs are now being considered. Obesity, while also frequently concomitant with asthma, appears to exacerbate the response to O_3 exposure exhibited by decreased FEV measurements and increased levels of inflammatory mediators (34-36). In addition, therapeutic studies using γ-tocopherol have already been shown to protect against air pollutants (37). Further, a potential impactful emerging extrinsic factor modifying susceptibility to O_3 are pharmacological agents. Our research demonstrates that APZ and other small molecule drugs alter the sterol concentration profile in airway epithelial cells, potentiating O_3 induced inflammation. By uncovering the susceptibility of the human airway to oxysterol generation and the tendency for drugs to modify available sterol ratios we have unveiled a new paradigm of study when looking at intensity of response to O_3 exposure, drug-x-environment interaction.

5.4.2 Emergent Knowledge Gaps

As stated, the data we present in previous chapters examining oxysterols and drug-x-environment interaction adds another possible clue to the responder/non-responder mystery. Our data provides evidence that O_3-derived oxysterols do have the capacity to increase inflammation in airway epithelial cells. Thus, pharmacological agents with either the designed or off target effect of modifying cholesterol biosynthesis can control the amount and ratio of oxysterol species being formed. Statins, drugs meant to inhibit HMG-CoA reductase at the beginning of cholesterol synthesis, have shown promise in some epidemiological studies in their ability to attenuate asthma and response to air pollution, however no conclusive clinical evidence has been reported (38,39). Statins could act to reduce O_3 induced inflammation by decreasing the pool of available cholesterol and presenting reduced levels of targets for ozonization during exposure. Whether statins can protect the airway from pollutants by altering the cholesterol constituency of the airway surface liquid and species oxysterols formed following O_3 exposure is worth exploring. Broadening our focus on the other widely used pharmaceuticals with off target effects inhibiting cholesterol synthesis enzymes like DHCR7 is prudent and it is currently unknown what combinations of drugs or pollutants could induce adverse effects. Inhibition of DHCR7 increases the levels of cholesterol precursor 7-DHC, a species more susceptible to ozonization and increasing the probability a highly reactive species will be formed. Thus, a current knowledge gap is whether there is a correlation between adverse health effects, high pollutant days, and pharmaceutical consumption.

5.4.3 Future Studies

In order to fulfill the new questions posed by our research, more intensive human pollutant exposure studies or epidemiological association studies need to be undertaken. Accordingly, O_3 exposure studies utilizing human volunteers should include the collection of data that could inform researchers of potential differences in cholesterol composition between individuals. For example, total cholesterol and sterol content could be evaluated in blood samples of volunteers collected pre exposure. Alternatively, IRB protocols could be developed to undertake a controlled O_3 exposure study in individuals taking APZ and compare them to individuals with no prescribed drug use. Consequently, drugs known to modify sterol levels, even as an off target effect, should be recorded in a subject's medical file, if not used as an exclusion criteria from the study, to allow for retrospective association studies and inform any O_3 response data. Second, a drug screening process, similar to what is undertaken studying potentially adverse chemicals with ToxCast™, can be used to reveal the potential for additional pharmaceuticals to alter cholesterol synthesis in various cell types, which could be considered for interaction with pollutants such as O_3. Instead of measuring toxicity, each drug or chemical could be screened for alterations in sterol profiles in the cell type of choice. Finally, drug x environment interaction studies could be undertaken through bioinformatics analysis and data mining of already existing air pollution and human response databases. Studies of this kind have previously evaluated geographic information systems considering ambient air pollution correlation with COPD hospitalizations in Jinan China (40). Similarly, the current status of machine learning algorithms, and new technologies in gathering pollution sensor data provide for large scale studies evaluating epidemiological data (41). UNC and North Carolina is a prime location to undertake such a study by assessing relationships between the thorough regional air quality monitoring, UNC hospital's records of adverse respiratory and cardiovascular events, and patient prescription histories. Considering the high percentage of Americans taking daily pharmaceuticals and the compounding factor that individuals already considered susceptible due to intrinsic factors may be prescribed various medications, our research may have defined a new archetype when evaluating environmental exposure response. The relationship between prescribed pharmaceuticals and environment should be considered in any new environmental exposure study or drug safety assessment.

5.5 Overall Impact

Evaluating the research described in previous chapters as a whole, the findings advance basic science regarding airway epithelial cell biology as well as provide new avenues for translational clinical research examining at respiratory health and air pollution. Although we have filled some of our originally proposed knowledge gaps, the major issues we have assessed will not be resolved by this research alone and there is much more to accomplish regarding the investigation of O_3 induced inflammation, oxysterols, NLRP2, and drug x environment interaction.

In my opinion, foremost among our findings is the potential for pharmaceutical drugs to interact with environmental pollutants. The ingredients for O_3 formation; atmospheric sunlight, heat, and volatile organic compounds (VOCs), are unlikely to decrease in the near future. Ground level O_3 levels in the U.S. much of Europe and Japan may have decreased over the past 25 years, however, marked increases in newly industrialized countries has increased the total global O_3 pollution (42). Compounding the growth of pollution, many of the countries with increasing industrial concerns, automobile exhaust, and other sources of VOCs exist near the equator and experience the highest temperatures and sunlight hours leading to massive creating of O_3 air pollution (43). Further, Climate change research indicates that any increase in global temperature may increase O_3 generation in the atmosphere (44). Although ground level O_3 serves as a well-established example to examine adverse health effects and drug interaction effects, other environmental pollutants may be additional relevant targets of interest to pursue. On a similar track, pharmaceutical drug use shows no signs of anything but exponential growth in the near future. The FDA alone approves approximately 50 novel drug formulations per year and studies have shown that almost 60% of the U.S. population takes at least one prescription drug (45). Taken together, the potential for drug-x-environment interaction may be the greatest threat to human safety in the United States.

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