The Cellular and Molecular Mechanism of Third Hand Cigarette Smoke-Induced Hepatic Steatosis

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

Cristina Flores

September 2017

Dissertation Committee:
    Dr. Manuela Martins-Green, Chairperson
    Dr. Frances Sladek
    Dr. Margarita Curra-Collazo
The Dissertation of Cristina Flores is approved:

__________________________________________________________________________

__________________________________________________________________________

__________________________________________________________________________

Committee Chairperson

University of California, Riverside
Acknowledgments

I would like to give special thanks my advisor and chair of my dissertation committee, Manuela Martins-Green and my other committee members, Francis Sladek and Margarita Curras-Collazo, for the helpful comments and suggestions on my work. I would also like to thank Kathy Redd, Patricia Springer, and Morris Maduro for their help, advice and support for these past years. I would also like to give special thanks to Star Lee for all her teaching advice and all my students for allowing me to serve as their teaching assistant. I would also like to thank the undergraduate students in the laboratory for their help in cutting the material for the cages to be exposed to THS. Lastly, I’m very grateful to the Tobacco Related Disease Research Program for funding these studies.

Dedication

This dissertation is dedicated to people I truly admire and love. Thank you for all your love and inspiration.
ABSTRACT OF THE DISSERTATION

The Cellular and Molecular Mechanism of Third Hand Cigarette Smoke-induced hepatic steatosis.

by

Cristina Flores

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, September 2017
Dr. Manuela Martins-Green, Chairperson

Recent evidence indicates that third hand smoke (THS) is present mainly in places where smoking happens regularly but also in places where smoking has been banned but previously happened regularly. THS toxins remain in the contaminated surfaces, are re-emitted and have the ability to react with atmospheric gases to produce dangerous carcinogenic compounds. New evidence regarding the toxicity of THS continues to emerge and has been accumulating for the past 5 years. In order to investigate effects of THS toxins in the liver, I took a molecular and cellular approach by performing multiple cellular assays to study important cellular processes involved in lipid metabolism. The rodent model I used mimics human chronic exposure to THS and separates the effects of
SHS (second hand smoke) from THS. I also used different drug treatments and antioxidant treatments to modulate the effects of THS in mice and determine whether oxidative stress plays a key role in the development of THS-induced hepatic steatosis. Using this cellular and molecular approach, I was able to show that THS toxins alter the oxidative stress levels and increase oxidative-stress-mediated damage (protein damage, DNA damage and functional damage) in mice resulting in abnormal lipid metabolism. I was also able to show regulatory pathways, which involve regulators such as AMPK, SIRT1 and SREBP1c, are altered by THS toxins.

Furthermore, THS toxins alter fatty acid metabolism and lead to a decrease in VLDL assembly contributing to accumulation of lipids in the liver. THS-exposed mice have high levels of TG and fatty acids and less ATP production, suggesting these animals are less efficient metabolically compared to the control mice. AICAR treatment resulted in a decrease of fatty acids and TG levels, suggesting that increasing Sirtuin 3 (SIRT3), a key regulator of fatty acid metabolism results in less lipid accumulation in the liver. These findings provide evidence of the cellular and molecular basis of THS-induced hepatic steatosis in mice.
# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... vi

LIST OF FIGURES ................................................................................................................ x

ACKNOWLEDGEMENTS ...................................................................................................... xi

INTRODUCTION .................................................................................................................... 1

CHAPTER 1: A brief overview of the effects of THS in human health ........................................ 4

Abstract ........................................................................................................................... 5

Introduction ....................................................................................................................... 7

Summary and Conclusion ............................................................................................... 13

References ......................................................................................................................... 14

Figures and Figure Legends ............................................................................................ 15

CHAPTER 2: Cellular and Molecular Mechanism of THS-Induced Hepatic Steatosis ............... 24

Abstract ........................................................................................................................... 25

Introduction ....................................................................................................................... 26

Material and methods ...................................................................................................... 31

Results ............................................................................................................................... 43

Discussion and Conclusion ............................................................................................ 49

References ......................................................................................................................... 52

Figures and Figure Legends ............................................................................................ 60
CHAPTER 3: The Role of Fatty Acid Metabolism and Apolipoproteins in THS-Induced Hepatic Steatosis in Mice

Abstract
Introduction
Material and methods
Results
Discussion and Conclusion
References
Figures and Figure Legends

CHAPTER 4: Conclusion
List of Figures

Figure 1.1 Schematic of long term THS exposure using mouse model.................19

Figure 1.2 Summary of major findings using mouse model to study the long-term
effects of THS toxins in different organs and behavior.................................21

Figure 1.3 Summary of the effects of THS on human health...........................23

Figure 2.1. THS exposure results in oxidative stress in the liver......................61

Figure 2.2. THS exposure increases oxidative stress mediated damage..............63

Figure 2.3. Antioxidant treatment leads to reduction of oxidative stress in the liver
of THS-exposed mice.................................................................65

Figure 2.4. Oxidative-stress-mediated damage is improved by treating the mice with
antioxidants.....................................................................................67

Figure 2.5. APAP treatment results in increase of oxidative stress in THS-exposed
mice..............................................................................................69
Figure 2.6. Treatment of THS-exposed mice with APAP increases oxidative-stress-mediated damage………………………………………………………………………………...71

Figure 2.7. THS toxins alter SIRT1/AMPK/ SBREP1c signaling in the liver………73

Figure 2.8. Proposed working model of THS-induced hepatic steatosis…………..75

Figure S1. Immunolabeling for lipid peroxidation…………………………………...77

Figure 3.1. THS-exposure affects FA, TG and total lipid levels in the liver………101

Figure 3.2. THS-exposure in conjunction with Western Diet does not affect FA, TG and total lipid levels in the liver…………………………………………………………………………………103

Figure 3.3. THS-exposure in conjunction with AICAR treatment affects FA, TG and total lipid levels in the liver…………………………………………………………………………………105

Figure 3.4. Effects of THS-exposure on the levels/activity of enzymes involved in fatty acid metabolism…………………………………………………………………………………107

Figure 3.5. THS toxins decrease IDH2 activity, SIRT3 and ATP levels in the liver of THS-exposed mice…………………………………………………………………………………109
Figure 3.6. THS exposure results in a decrease of VLDL assembly in the liver.....111

Figure 3.7. Plausible mechanism of how THS toxins alter VLDL assembly and fatty acid breakdown in the liver resulting in THS-induced hepatic steatosis..............113
Introduction
In 1881 James Bonsack invented the cigarette-making machine leading to the widespread smoking of cigarettes. For more than a century people have been smoking commercial cigarettes around the world resulting in many tobacco-related deaths each year. The adverse effects of smoking and second hand smoke (SHS) on human health have been shown via many research studies over the last decades. Only recently has the term third hand smoke (THS) been coined and introduced into the field of tobacco research. Third hand smoke is defined as the layer of tobacco toxin residues that accumulates on surfaces where smoking has occurred and/or continues to occur. THS has the following characteristics: (1) remains on surfaces; (2) re-emits into the gas phase; (3) reacts with other molecules present in the environment, resulting in the formation of carcinogens. Studies supporting the existence of THS have been accumulating over the last decade and have confirmed the presence of nicotine, one of the major components of THS, in the homes of smokers from various regional and socioeconomic demographics, suggesting exposure to toxins among different sectors of society. However, the effects of THS in human health have not been widely studied and the toxicity remains largely unknown.

Among the challenges that researchers face when studying the effects of THS in human health, is the low number of human subjects. Also, it is very difficult to control how many people smoke and consequently the exposure levels vary among subjects. Our lab has designed an *in vivo* system to study the systemic effects of long-term THS exposure using mice as the animal model. This system is ideal because of the following
reasons: (1) It differentiates between second hand smoke and THS; (2) one can perform controlled exposures; (3) it allows for the use of many animals so that statistical analysis can be performed.

In this document I start by reviewing what is currently known about THS toxicity (Chapter 1). Then I proceed to show molecular and cellular mechanisms of THS-induced hepatic steatosis (Chapter 2). Subsequently, I discuss the role of THS-exposure on fatty acid metabolism and on lipoproteins in hepatic steatosis (Chapter 3). Lastly, I conclude by summarizing the findings and discussing the implications of our findings for liver health (Chapter 4).
CHAPTER 1:
A brief overview of the effects of THS in human health
Cigarette toxins are detrimental to human health because these toxins affect a wide variety of biological processes. The smoke is divided into active SHS and passive THS. SHS and THS co-exist initially when THS is starting to form from the tobacco toxins. Unlike SHS that is visible, THS is invisible and consequently the characterization of THS toxins is not yet fully developed. Unlike SHS exposure, which occurs in low intervals at high dose mainly via inhalation, the exposure to THS exposure is slow and at low dose for prolonged periods and can occur via inhalation, ingestion and dermal uptake. These toxins can remain on these surfaces for days or months. If the exposure to THS is chronic, multiple layers of toxins build up continuously making these surfaces very toxic. These toxins age with time, becoming progressively more reactive and toxic. THS toxins have the ability be re-emitted and react with other gaseous compounds found in the atmosphere and lead to the formation of tobacco specific N-nitrosamines (TSNA) such as 1-(N-methyl-N-nitrosamino)-1-(2-pyridinyl)-4- butanal (NNA), 4-(methylnitrosamino)-1-(3-pyridinyl)-1-buta- none (NNK) and N-nitroso nornicotine (NNN). These chemicals are carcinogens. Two specific TSNA, NNA and NNK have been shown to result in genotoxicity and result in DNA damage. *In vivo* and *in vitro* studies have been done to study the effects of THS in human health. Multiple epidemiological studies have shown the presence of chemical metabolites in the urine of children who live in the houses of smokers. The chemical metabolites also have been found in the homes of families who reported no indoor smoking. It is now known that THS exposure results in chemical assimilation but the repercussions of this exposure still remain to be elucidated. Recent studies also showed that THS exposure leads to abnormal
cognitive functions in children and DNA damage in human cells [1-2]. Mice exposed to THS toxins also show higher levels of hyperactivity compared to mice that are not exposed to THS.

In recent years, the effects of third hand smoke (THS) in human health have been investigated using different experimental approaches ranging from epidemiological toxicological studies to biological studies. Epidemiological and toxicological studies showed THS toxins remained, were reemitted and reacted with atmospheric gasses leading to the formation of carcinogenic compounds leading to additional research studies [3-9]. These studies also showed that THS toxins could be absorbed, metabolized and excreted in the urine [10-12]. Using cell lines, researchers have been able to show THS exposure results in DNA damage and changes in gene expression \textit{in vitro} [13-20]. The animal studies have provided more insights into the effect of THS toxins in immunity and metabolism [21-22]. In this chapter, I discuss a brief overview of the research progress in the field of THS with a specific focus on the studies that have investigated the effects of THS exposure in human health.

**THS exposure in epidemiological human studies**

The amount of evidence of the toxicity of third hand smoke has been accumulating over the past decades. Children are among the populations more susceptible to toxicity of THS since they spend more time indoors and continue to develop through their childhood. Children also have the tendency to crawl and touch contaminated
surfaces more frequently than adults; consequently they come into close contact with THS toxins more often.

In a recent study, Matt et al. was able to show that nicotine levels were higher in the houses of smokers than nonsmokers and that nicotine metabolites were higher in children living in houses of smokers than nonsmokers [3]. By measuring the (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) (NNAL)/cotinine concentrations in infants’ urine, the researchers were able to show neonatal intensive care units are contaminated with THS toxins [12]. In another study, Ramirez et al. measured the nicotine and nitrosamines/TSNAs levels in household samples and found the cancer risk for children increased [13]. Some of the THS toxins can act as neurotoxic and low levels of prenatal exposure results in cognitive deficits in infants [1].

**THS exposure results in genotoxicity in cell models**

THS contains many toxic compounds, which include mutagens and carcinogens resulting in genotoxicity. Genotoxicity is one of the critical mechanisms responsible for many types of cancer caused by active smoking and SHS exposure. The ability of THS to alter the genome integrity of humans was not well known but recent research studies have been done to access the genotoxic potential of THS toxins. The main finding of these studies is summarized below.

In a recent study, significant increases in DNA strand breaks were observed in human liver cancer cells (HepG2) exposed to THS [15]. They discovered both acute and chronic exposure of materials to THS-induced DNA strand breaks, suggesting that THS
toxins are genotoxic because double strand breaks (DSB) are the most dangerous form of DNA damage. Another study demonstrated that when epithelial cells (BEAS-2B) were exposed to THS toxins, double strand breaks formed. Interestingly, the amount of DSB formed was similar to the amount of DSB induced by SHS [16]. Other studies have investigated the effect of specific THS constituents, such as NNK and NNA, on DNA stability and have shown increasing the dose exposure of these toxins leads to DNA damage [16-17]. Low amounts of 0.01 to 100 µM of NNA resulted in DSB in HepG2 cells [16].

**THS exposure results in metabolic changes in male cell line**

Recent studies have shown THS exposure alters the metabolome of cell lines suggesting the ability of THS toxins to alter the metabolism of these specific cell lines. Chronic exposure of GC-2 and TM-4, two rodent cell lines, resulted in changes in cell cycle, apoptosis, reactive oxygen and glutathione metabolism in GC2 whereas in TM-4, ammonia metabolism was altered [20]. In this study, using RT-PCR analyses, they were able to show that enzymes involved in glutathione, nucleic acids and ammonia metabolism, were changed.

**THS exposure results in changes in body mass and immunity in mice**

Some studies have been done accessing the health threat of THS to infants and young children who are in smokers’ homes and discovered that the damage that occurs at early stages may continue in adulthood. In this study, they exposed C57BL/6J mice to THS and investigated the effects of THS exposure on body mass and blood cell
population from birth until weaning (21 days old). Both THS-exposed female and THS-exposed male mice had a significant reduction in weight gain compared to the non-exposed mice. However, the effect of THS on body mass was diminished after the mice were placed in a non-exposed setting. The THS-exposed mice displayed similar body mass to the control mice at 5, 8, 12 weeks [21].

THS exposure during the first 3 weeks of life altered the immunity in the THS-exposed mice. THS exposure resulted in an increase of eosinophil number in blood circulation in both genders at 17 weeks of age. The-exposed male mice also had a significant increase in basophil levels and THS-exposed mice had increased neutrophils. Fluorescence-activated cell sorting (FACS) analysis in the blood of these mice also showed THS exposure results in a significantly increased percentage of B-cells and T-suppressor cells, with a decreased percentage of myeloid cells in adult rats [18]. Taken together these findings suggest early exposure to THS results in cellular damage and that this damage can persist into adulthood.

**THS exposure results in changes in metabolic and behavioral changes in mice**

The studies summarized above focused on dose-dependent damage and acute exposure. Studies investigating the effects of long-term exposure have also been performed in our laboratory to study the effects of THS in different organs. These studies were done and are being done using a rodent model, which separates the effects of SHS from THS exposure and mimics the exposure of THS toxins in humans. The mice live in
cages of exposed material (10g curtain, 10g carpet and 10g upholstery) for six months before specific analyses are done (Figure 1.1). The materials are exposed to THS toxins using a smoking machine (Teague Enterprises, Inc., Woodland, CA) and two packs of 3R4F research cigarettes are smoked each day, 5 days/week [23-24]. After six months of exposure the effects of THS toxins in specific organs and behavior are investigated and the main findings are summarized below (Figure 1.2).

**THS exposure results in inflammation and fibrosis in the lung of mice**

Exposure to THS-toxins for six months resulted in an increase of leukocyte infiltration to respiratory bronchioles suggesting chronic exposure to THS toxins leads to inflammation in the lung. The alveoli of the THS-exposed mice were also disrupted and high amounts of disorganized collagen fibers were present in the lung tissue indicating fibrosis [22]. Taken together these findings suggested that THS exposure could result in the development of lung fibrosis in mice.

**THS exposure results in altered behavior in mice**

Chronic exposure to THS-toxins results in hyperactivity in THS-exposed mice. An Open Field test analysis of these animals showed that mice exposed to THS toxins had the tendency to move more than the control and transition less between behaviors (walking, stationary or rearing). Consequently THS-exposed mice covered longer lengths
at higher speeds of movement than the control mice. THS-exposed mice also had the tendency to stay in the periphery more than the control mice. Taken together, the open field analysis indicated that THS toxins have the ability to alter the behavior and result in hyperactivity in mice.

**THS exposure result in abnormal lipid and glucose metabolism in mice**

Chronic exposure to THS toxins results in abnormal lipid metabolism in the liver. THS-exposed mice accumulated higher levels of lipids in the liver resulting in hepatic steatosis in male mice. Histological analysis of liver tissue of THS-exposed mice showed that these mice had larger lipid droplets compared to the control [22]. Circulating triglycerides (TG) and low-density lipoprotein (LDL) were also elevated whereas high-density lipoprotein (HDL) was decreased in THS-exposed mice compared to the control. Elevated levels of TG and LDL have been associated with metabolic syndrome and cardiovascular diseases [25-35]. Therefore, THS-exposed mice seem to be predisposed to cardiovascular diseases and type 2 diabetes [22]. Exposure to THS-toxins resulted in prediabetic phenotype in THS-exposed mice. These animals had elevated fasting glucose compared to the control. THS exposed mice were unable to metabolize glucose or use insulin efficiently when challenged with glucose using the IPTG test or insulin (IIT) test. Taken together, these findings suggested that chronic exposure to THS toxins has the ability to induce hepatic steatosis in mice. These findings have a significant clinical relevance because smoking has been linked to abnormal glucose metabolism in humans in previous studies [36-42].
Summary and conclusions

Evidence is mounting for the past seven years that THS toxins are damaging to the body. Epidemiological, toxicological, in-vitro and animal research studies have demonstrated that THS exposure can have detrimental effects in human health (Figure 1.3). More specifically, this evidence has demonstrated THS toxins have the ability to alter the following: (1) DNA integrity (2) behavior (3) metabolism and (4) immunity. The mechanism for which this cellular and molecular damage occurs is not yet fully understood and additional studies need to be done to elucidate how THS-toxins can induce this damage.
References


37. Royal College of Physicians. Passive smoking and children. A report of the


Figure 1.1 Schematic of long term THS exposure using mice model. Mice (3 weeks of age) are exposed to THS toxins for six months. Common household material (10g carpet, 10g cotton and 10g upholstery) is exposed to THS toxins a Teague Smoke Machine that mimics a interment smoker (2 packs of cigarettes each day for 5 days).
Figure 1.1

THS exposure system in mice

Expose material to THS toxins

- 10g Curtain
- 10g Cotton
- 10g upholstery

Expose mice (3 weeks of age) to THS toxins for 6 months

Analysis of the toxicity of THS toxins

- Effects on immunity
- Effects on organ function
- Effects on metabolism
Figure 1.2 Summary of major findings using mice model to study the long-term effects of THS toxins in different organs and behavior. THS exposure results in changes in behavior, lung damage, lipid accumulation in the liver and altered glucose and lipid metabolism in mice.
Summary of long-term effects of exposure to THS in mice

- Inflammation
- Slow wound healing
- Abnormal glucose metabolism
- Fibrosis in the lung
- Hyperactivity
- Hepatic steatosis

Figure 1.2
Figure 1.3 Summary of the effects of THS on human health. THS toxins results in DNA damage, changes in metabolism, changes in immunity and altered behavior in mice.
Findings of the toxicity of THS toxins from *in vitro* studies

- DNA damage
- Changes in metabolism
- ROS production

Effects of THS toxins in human health

Findings from toxicity of THS toxins from *in vivo* studies

- Changes in immunity
- Altered organ function
- Changes in metabolism
- Changes in body mass
- Changes in behavior
- Inflammation and fibrosis

*Figure 1.3*
CHAPTER 2:
Cellular and Molecular mechanism of THS-induced hepatic steatosis
Abstract

**Background:** Third hand smoke (THS) forms when second hand smoke (SHS) tobacco toxins accumulate on surfaces such as walls, carpets and clothing and can result in adverse health effects.

**Objective:** This study was designed to investigate the mechanism of THS-induced hepatic steatosis.

**Methodology:** We used an *in vivo* exposure system that mimics exposure of humans to THS to investigate the effects of THS on hepatic lipid metabolism. THS-exposed mice were treated either with the liver-damaging drug, N-acetyl-p-aminophenol (APAP/Tylenol) to increase oxidative stress or with the antioxidants N-acetyl-cysteine and α-Tocopherol which decrease oxidative stress.

**Results:** THS-exposed mice have higher levels of superoxide dismutase activity and H$_2$O$_2$ levels. However, no significant changes in activity of the antioxidant enzymes catalase and glutathione peroxidase were found, implying the presence of high levels of hydrogen peroxide in the liver. Furthermore, THS-exposed mice also have a lower NADP$^+$/NADPH ratio, indicating decreased ability of these mice to combat oxidative stress. THS-exposed mice show a decrease in ATP production, increase in aspartate aminotransferase (AST) activity, as well as increased molecular damage (lipid peroxidation, protein nitrosylation and DNA damage). Treating THS-exposed mice with APAP/Tylenol enhances the THS-induced damage whereas treating with antioxidants reduces the damage. THS-exposed mice also have lower sirtuin 1 (SIRT1) levels compared to controls which decreased activation of 5' AMP-activated protein kinase (AMPK) and increased sterol regulatory element binding protein 1c (SREBP1c).

**Conclusion:** THS-exposed mice on a normal diet have increased oxidative stress and damage mediated by oxidative stress, which results in alterations to the SIRT1/AMPK/SREPB1c signaling pathway. Increasing oxidative stress results in enhanced THS-induced damage whereas decreasing oxidative stress causes improvement in the THS-induced liver damage. Our results show that THS is a new risk factor contributing to the development of liver steatosis and highlight the danger of THS in general.
Introduction

Cigarette toxins are detrimental to human health; these toxins affect a wide variety of biological processes. The cigarette toxins are initially emitted when the smoker exhales the smoke of the cigarette into the air as mainstream second hand smoke (SHS); these toxins will then transform physically and chemically, giving rise to third hand smoke (THS). SHS smoke is the smoke that is inhaled and exhaled by the smoker and by a by-stander directly. The exposure frequency of SHS is short intervals at very high dosage and then, with time, SHS toxins dissipate and are removed by ventilation. However, SHS has been shown to be very dangerous to non smokers and numerous studies have shown its effects in human health [1-2]. Second hand smoke contains more than 4000 chemicals, which include ammonia, carbon monoxide and sulfur dioxide [1-2]. There is a strong correlation between second hand smoke and lung cancer in both the smoker and non-smoker [2]. Some studies have highlighted the dangers of second hand smoke to non smokers by showing it results in 50,000 deaths per year [2]. The effects of SHS have been widely studied and many publications have addressed the damage of SHS toxins in multiple organs via oxidative stress [3-9].

Third hand smoke (THS) forms when second hand smoke (SHS) tobacco toxins accumulate on surfaces such as walls, carpets and clothing [10-13]. Moreover, these toxins have the ability to react with gases in the air, such as nitrous acid, and produce dangerous carcinogens [14-18]. Individuals are exposed to THS toxins when they come into contact with the deposited toxins or when the toxins are re-emitted into the air. Exposure to THS toxins can result in detrimental health outcomes in individuals who are
chronically exposed to them [19-26]. These health effects vary from metabolic disorders to cognitive perturbations. Children are one of the sectors of the population that is most susceptible to tobacco toxins and studies have shown that tobacco metabolites are detected in their urine [22-24]. Unfortunately, this dangerous exposure continues. In 2007, about 5.5 million children lived in households where someone smoked inside the home [21]. Even in households where indoor smoking was not reported, the tobacco metabolites could still be detected in the children’s urine [22-25].

Studies *in vitro* have shown that THS is toxic to human cells. When a liver cell line, HepG2 cells, was exposed to THS toxins their DNA integrity and stability was compromised [25]. Both acute and chronic THS exposure resulted in double strand DNA breaks [25]. These investigators also showed that the molecular damage observed in HepG2 cells was associated with the increase in cellular oxidative stress and therefore suggested that oxidative stress is a major contributor to THS-mediated toxicity *in vitro* [25]. Another *in vitro* study focused on the effects of THS in the development of the lung using fetal rat lung explants [26]. These investigators showed that when fetal rat lungs were exposed to the THS toxins nicotine, 1-(N-methyl-N-nitrosamino)-1-(3-pyridinyl)-4butanal (NNA), or 4-(methylNitrosamino)-1-(3-pyridyl)-1-butanone (NNK), there was a disruption in the development of alveolar interstitial fibroblasts and that the disruption occurred via down regulation of PPAR-γ signaling [26]. PPAR-γ signaling controls alveolar development and down regulation of this signaling in the lungs suggested a disruption in alveolar epithelial-mesenchymal paracrine signaling, which prevents normal lung development. Exposure to THS toxins also resulted in increase of fibronectin and
calponin protein levels as well as increase in apoptosis, which are markers for abnormal lung development. Together these findings suggest that the lungs of rats exposed to THS toxins do not develop normally, highlighting the danger of exposure to these toxins during lung development.

It also has been shown that THS toxins have the ability to cause metabolic changes in two male germ cell lines [27]. When spermatogonia (GC-2) and sertoli derived cells (TM-4) were exposed to THS toxins for 24 hours, the investigators observed changes in metabolic enzymes involved in glutathione, ammonia and nucleic acid metabolism [27]. In GC-2 cells, THS exposure resulted in increase in synthesis and decrease in the breakdown of GSH. GSH is a major component of the antioxidant defense pathway and these findings suggest spermatogonia might use this enzyme to combat the high levels of oxidative stress [27]. In TM-4 cells, there are increased metabolites involved in nucleic acid metabolism, suggesting that THS toxins are genotoxic and have the ability to compromise the integrity of genetic material [27]. THS exposure did not have effects on cell viability, cell cycle or apoptosis in these two male germ cell lines [27]. However, this in vitro study highlighted the sensitivity of male germ cells to THS toxins.

In addition to the findings in vitro, the detrimental effects of THS toxins have also been shown to alter human behavior. Epidemiological studies have shown that THS exposure results in abnormal cognitive function in children [28]. Children exposed to THS toxins are more hyperactive than children that live in environments with less THS contamination. Also, children are exposed to higher levels of these toxins than adults.
because they are in closer contact with the contaminated surfaces, lick their fingers and their surface-to-volume ratio is larger [28-29]. In addition, they are also more susceptible because their liver is not fully developed and therefore is not as effective in detoxification of toxins. Also, smoking during pregnancy or after pregnancy is dangerous for the developing fetus and results in impaired lung function [30-31]. Post-natal exposure to THS toxins also results in increased problems with the larynx, trachea and bronchi. This resulted in increased cough-and-asthma related symptoms in these children, suggesting THS toxins are detrimental for lung health of children living in homes contaminated with THS toxins.

We have developed an in vivo THS exposure system of mice that mimics THS exposure in humans [32]. Our model is a novel exposure system that allows us to study the effects of long-term THS exposure in different organs of male C57BL/6 mice. Using this exposure method, we have shown that THS exposure leads to lipid accumulation in the liver of these mice, suggesting that they have abnormal metabolism and insulin resistance [32-33]. These findings suggest that THS exposure is a new risk for hepatic steatosis.

Hepatic steatosis is the earliest stage of and a hallmark of non-alcoholic liver disease (NAFLD), which is the most common liver disease worldwide [34-35]. In some patients, steatosis can progress into fibrosis and then to cirrhosis if is not treated early. In the U.S., the prevalence of NAFLD is estimated to be around 20% in the general population and the percentage quadruples in morbidly obese individuals [34-35]. NAFLD is not limited to adults; the incidence of NAFLD among the pediatric population is
increasing and it is estimated to account for about 14% of all NAFLD cases [34-35].
NAFLD is a complex process that results from metabolic abnormalities [36-37]. Many patients show dyslipidemia, malfunction of beta oxidative enzymes, deficiency in lipoproteins, and immune dysregulation [38-39]. Obesity, metabolic disorders, drugs or smoking are some of the major risk factors contributing to the development of NAFLD [40-42]. This disease is primarily controlled through changes in life-style, cessation of chemical exposure or antioxidant treatments [43-45].

In this study, we performed cellular and molecular studies to investigate whether THS exposure leads to increased oxidative stress in liver, leading to abnormal lipid metabolism and resulting in hepatic steatosis. Here, we show that mice exposed to THS toxins have altered oxidative stress in the liver. Increasing oxidative stress by treating the mice with N-acetyl-p-aminophenol (APAP/tylenol) leads to exacerbation of THS-induce hepatic steatosis whereas antioxidant treatment ameliorates this condition. These findings suggest that oxidative stress caused by THS toxins leads to lipid accumulation in the liver. More specifically, THS toxins alter the SIRT1/AMPK/SREPB1c pathway, which regulates lipid metabolism in the liver, suggesting, that THS toxins are a new risk factor for development of hepatic steatosis.
Materials and methods

Animals: Male C57BL/6 mice were divided into control and experimental groups (THS-exposed mice). The experimental group was exposed to THS toxins after weaning for 24 weeks. The control group was never exposed to THS toxins. Both control and THS-exposed mice were maintained under controlled environmental conditions -- 12-hr light/dark cycle in conventional cages with *ad libitum* access to standard chow (percent calories: 58% carbohydrate, 28.5% protein, and 13.5% fat) and water.

Ethics Statement: All animal experimental protocols were approved by the University of California, Riverside, Institutional Animal Care and Use Committee (UCR-IACUC). Mice were euthanized by carbon dioxide (CO₂) inhalation, which is the most common method of euthanasia used by NIH. The amount and length of CO₂ exposure were approved by UCR-IACUC.

THS exposure method: Common household fabrics such as curtain material, upholstery and carpet were placed in empty mouse cages and exposed to SHS generated by the a Teague smoking machine as previously described in [32]. Each cage contained 10 g of curtain material (cotton) 10 g of upholstery (cotton and fiber) and two 16in² pieces of carpet (fiber) to maintain equal exposure levels across all experimental groups. Two packs of 3R4F research cigarettes were smoked each day, five days per week to mimic an intermittent smoker. The smoke was routed to a mixing compartment to mix with air and
the mixture distributed between two exposure chambers, each containing eight cages with
the materials. The gravimetric method was used to determine the total particulate
concentrations in each smoke chamber. The weight of Whatman grade 40 quantitative
cellulose filter papers was recorded before introducing the filter paper into the filtering
device and the device was then allowed to run for 15 minutes. After 15 minutes, the filter
was weighed again to determine the particulate mass that had accumulated during this
time period. This procedure was repeated with two more filters and the average of the
three masses gave the TPM values for each chamber. The TPM values were adjusted to
30 µg/m³, which resembles the values found in the homes of smokers by the EPA.
All cigarettes were smoked and stored in accordance with the Federal Trade Commission
(FTC) smoking regimen. At the end of each week, cages were removed from the
exposure chamber, bagged, and transported to the vivarium where mice were placed into
the cages with the THS exposed material. For the next week, an identical set of cages and
fabric was prepared and exposed to smoke in the same way as the first set of cages. By
using two sets of cages and materials, each of which was exposed on alternating weeks,
we ensured that mice inhabited cages containing fabric that had been exposed to fresh
SHS in the beginning of each week and aged smoke towards the end week. Throughout
the exposure period, hair was removed from the backs of the exposed mice (and controls)
to mimic the bare skin of humans.

**N-acetyl-p-aminophenol (APAP/tylenol) treatment:** Four cohorts of male mice of the
same age were used for *in vivo* APAP treatment studies. APAP was purchased from
Sigma (Cat# A5000-100G) and was dissolved in warm PBS and injected IP. THS-exposed mice and controls received a daily dose of 300 mg/kg for eight weeks via IP injections. The concentration of each APAP solution was adjusted so that all mice received approximately the same volume.

**Antioxidant Therapy:** Four cohorts of male mice, control and THS-exposed, were given antioxidants daily starting at weaning (3 weeks of age) for five months. We used N-acetylcysteine (NAC) and α-tocopherol (α –TOC). NAC (50 mg/kg) was dissolved in PBS and injected daily via IP. α-TOC (150 mg/kg) was dissolved in 70% ethanol and also injected IP daily.

**Tissue Extracts:** After six months of THS exposure, mice were sacrificed livers were extracted from THS-exposed or control mice, washed with PBS and immediately frozen. The frozen livers were stored at -80°C. Liver tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA), centrifuged, and the supernatant collected unless otherwise specified by instruction manuals of the assay kits used in this study. Extracts were used to perform the various assays we used for this study.

**Blood Extracts:** Blood was extracted directly from the heart and allowed to coagulate on ice for 20-30 minutes. The samples were then centrifuged at 10,000 rpm for phase separation. The serum was used immediately for assays or frozen and stored at -80°C.
and later used in assays.

**Tissue embedding and Immunolabeling:** Liver tissues from control and THS-exposed mice were fixed in 4% paraformaldehyde overnight, then rinsed in 1X PBS, incubated in 0.1 M glycine solution, followed by an incubation with 15% sucrose and subsequently 30% sucrose solution and embedded in optimal cutting temperature (O.C.T.) embedding medium (Cat# VWR 25608-930). Sections 8 µm thick were prepared and the tissue immunolabeled for 4-HNE, a marker for lipid peroxidation. Sections were blocked with 10% BSA, then incubated with mouse anti-HNE antibody (1:100) (Abcam; Cat # ab48506) overnight at 4°C, the excess primary Ab washed and the sections incubated with AlexaFluoro 568-labeled goat anti-mouse IgG antibody (1:3000) (Invitrogen; Cat# A-11004). The excess of secondary antibody was washed away, the sections mounted with Vectashield and imaged using a Nikon Microphot-FXA fluorescence microscope with a Nikon DS-Fi1 digital camera and Nikon NIS-Elements software (Nikon Instruments Inc., Melville, NY).

**Measurement of SOD activity:** Cayman Superoxide Dismutase Assay Kit (Cat # 706002) was used to measure SOD levels in the liver. Frozen liver pieces (10 mg) were placed in ice-cold RIPA buffer containing 50mM Tris (50mM pH 7), NaCl (150mM), SDS (0.1%), sodium deoxycholate (0.5%) and Triton X (1%) and homogenize using a Bullet Blender Homogenizer to extract total protein. The samples were centrifuged at 10,000 rpm for 15 min at 4°C, and the resulting supernatant was used for SOD activity.
assay. Protein quantification was done using the Bradford assay (Bio-Rad). SOD activity was quantified by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. A standard curve was generated and used to quantify the activity of SOD in the liver samples.

**Measurements of hydrogen peroxide levels:** A Cayman Hydrogen Peroxide Assay Kit (Cat # 600050) was used to measure H$_2$O$_2$ levels in the liver. Fresh liver samples (10 mg) were homogenized in ice-cold RIPA buffer containing 50mM Tris (50mM pH 7), NaCl (150mM), SDS (0.1%), sodium deoxycholate (0.5%) and Triton X (1%) using a Bullet Blender Homogenizer to extract total protein. The samples were centrifuged at 10,000 rpm for 15 min at 4°C, and the resulting supernatant was collected for the assay. H$_2$O$_2$ was detected using 10-acetyl-3,7-dihydroxyphenoxazine (ADHP), a highly sensitive and stable probe for H$_2$O$_2$. In the presence of horseradish peroxidase, ADHP reacts with H$_2$O$_2$ in a 1:1 stoichiometry ratio to produce a highly fluorescent resorufin (excitation = 530-560 nm; emission = 590 nm). The standard curve generated was used to quantify the activity of hydrogen peroxide in the liver.

**Measurement of catalase activity:** Cayman Catalase Assay Kit was used to measure catalase (Cat # 707002) activity. Frozen liver pieces (10 mg) were placed in ice-cold RIPA buffer containing 50mM Tris (50mM pH 7), NaCl (150mM), SDS (0.1%), sodium deoxycholate (0.5%) and Triton X (1%) and homogenized using a Bullet Blender Homogenizer to extract total protein. The samples were centrifuged at 10,000 rpm for
15 minutes at 4°C, and the resulting supernatant was collected and used for the assay.

20µl of tissue homogenate were added to the wells of a 96 well plate along with 100µl of assay buffer and 30µl methanol and the reaction initiated by addition of 20µl H2O2. After incubation for 20mins, 30µl of potassium hydroxide was added to terminate the reaction. 30µl of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald), which is a chromogen, was added. After incubation for 10 minutes at RT, 10µl potassium periodate was added and incubated again at RT for five minutes. Absorbance was read at 540nm. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes which, upon oxidation, changes from colorless to a purple color. The amount of catalase activity levels were calculated and standardized for protein using the Bradford method (Bio-Rad).

**Measurements of GPx activity:** Cayman GPx activity assay kit (Cat# 703102) was used to measure GPx (Cat # 703102) activity in the liver. Frozen liver pieces (10 mg) were homogenized in ice-cold RIPA buffer containing 50mM Tris (50mM pH 7), NaCl (150mM), SDS (0.1%), sodium deoxycholate (0.5%) and Triton X (1%) using a Bullet Blender Homogenizer to extract total protein. The samples were centrifuged at 10,000 rpm for 15 minutes at 4°C, and the resulting supernatant was collected and used for the assay. 20µl of liver homogenate were added to the wells of a 96 well plate along with 100µl of assay buffer and 50µl of co-substrate composed of NADPH, glutathione and
glutathione reductase. Reactions were initiated by addition of hydroperoxide, mixed for a few seconds, and absorbance was read at 340nm every minute to obtain at least 5 time points. The assay measured GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPx, was recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ was accompanied by a decrease in absorbance at 340nm. The rate of decrease in the A340 was directly proportional to the GPx activity in the liver homogenate samples.

**Measurement of DNA damage:** DNA damage was measured using an ELISA Kit from Cell BioLabs Inc (Cat# STA-320) which allows the quantification of 8-hydroxy-2-deoxy Guanosine (8-OH-dG), DNA damage marker in ng/ml. DNA was extracted from liver samples, dissolved in water (1-5 mg/mL) and converted into single stranded DNA by incubating the sample at 95°C for five minutes following by chilling on ice. DNA was digested to nucleosides by incubating the denatured DNA with 5-20 units of nuclease P1 for 2 hours at 37°C at final concentration of 20 mM Sodium Acetate, pH 5.2, followed by treatment with 5-10 units of alkaline phosphatase for 1 hr at 37°C in a final concentration of 100 mM Tris, pH 7.5. The reaction mixture was centrifuged for five minutes at 6,000 rpm and the supernatant used for the 8- OH-dG ELISA assay. 50 µL of experimental samples or 8-OHdG standards were added to the wells of the 8-OHdG Conjugate coated plate. Incubation was performed at room temperature for 10min on an orbital shaker. 50 µL of anti-8-OHdG antibody was added to each well and the plate was incubated at room
temperature for 1hr. The microwells were then washed three times with Wash Buffer. After the last wash, 100 µL of secondary Antibody-Enzyme Conjugated was added and incubation was performed at room temperature for 1 hr followed by three washes. 100 ul of Substrate Solution containing 3,3’, 5,5’Tetramethylbenzidine was added to each well and incubated at room temperature for 30 minutes. The enzyme reaction was stopped by addition of 100 µL of Stop Solution into each well. Absorbance was read on a spectrophotometer at λ=450 nm.

**Measurement of nitrosylation of proteins:** Protein damage was quantified by measuring the nitrosylation of tyrosine residues in proteins. The amount of 3-nitrotyrosine in the liver proteins was determined using the OxiSelect Nitrotyrosine ELISA Kit from Cell Biolabs (Cat# STA-305). The liver samples or nitrated BSA standards were first added to a nitrated BSA pre-absorbed enzyme immunoassay (EIA) plate. After a brief incubation, an anti-nitrotyrosine antibody was added, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in the liver samples were determined by comparing with a standard curve that was prepared from predetermined nitrated BSA standards provide in the kit.

**Measurement of lipid peroxidation:** Lipid peroxidation in the liver was determined by the levels of the byproduct of lipid peroxidation known as malaldehyde (MDA) using the OxiSelect TBARS Assay Kit from Cell Biolabs (Cat # STA-330). The unknown MDA containing samples or MDA standards were first reacted with Thiobarbituric Acid (TBA)
at 95°C for 60 minutes. After this incubation period, the plate containing the samples and standards was read fluorometrically at 540 nm excitation and 590 nm emission. The MDA content in unknown samples was determined by comparison with the predetermined MDA standard curve.

**Measurement of AST levels:** The AST levels were measured using the Aspartate aminotransferase (AST) Activity Assay (Cat# MAK055). 100 uL of the reaction mix (80 uL AST Assay buffer; 2 uL AST enzyme mix; 8 uL AST developer; 10 uL AST substrate) was added to each well. The plate was then mixed by pipetting and incubated at 37°C and was protected from light. The first initial reading was taken after 2 minutes of incubation. After the recording of the first initial reading, the subsequent readings where taken every five minutes until the measurements of the samples where greater than the value of the highest standard (~10nmole/well). The background in the wells was corrected by subtracting the final reading of standard zero from all the final readings of the eight standards. The standards were then used to make a standard curve. The AST activity was calculated by subtracting the final reading of each sample from initial reading divided by the reaction rate and volume of the sample.

**Measurement of ATP levels:** The ATP levels in liver were quantified using BioVision’s ATP Colorimetric and Fluorometric Assay kit (Cat# K354-100), which is designed to detect low quantities of ATP (~50pmol) by measuring the phosphorylation of glycerol. The assay was performed according to the protocol included in the kit. Liver
tissue samples (10mg) were homogenized in 100 µl of ATP assay buffer and 50 µl of sample supernatant was added to a 96-well plate in duplicates. 50 µl of the Reaction Mix (44 µl ATP Assay buffer; 2µl ATP probe; 2 µl ATP converter; and 2 µl developer) was added to each well containing the ATP standards and samples to initiate the phosphorylation of glycerol reaction. The plate was mixed well and incubated for 30 minutes at room temperature away from light. During this incubation period, glycerol was phosphorylated. The product of this phosphorylation reaction was read at λ=570nm and the absorbance readings of the standards were used to generate a standard curve. The unknown amount of ATP in the samples was then calculated using the standard curve and the observations reading of the samples.

**Measurement of Urea levels:** Urea levels in the liver tissue were quantified using Cell Biolabs’ Urea Assay Kit (Cat # sta-382), which is based on the Berthelot reaction. The enzyme urease is used to catalyze the hydrolysis of urea into carbon dioxide and ammonia. Ammonia then reacts with the alkaline developer (Berthelot’s reagent) included in this kit to produce a blue-green colored product that can be measured with a standard spectrophotometric plate reader at an optical density 630nm. 10 µl of the standards or samples were added to the 96-well plate. 100 µl of the urease/Ammonia Reagent (40 mg of urease for 10 mL of Ammonia reagent) mixture was added to each well. The plate was mixed and incubated for 10 minutes at 37°C. After incubation, 100 µl of the Developing Reagent was added to each well using a multichannel pipette. The
plate was placed on an orbital shaker to mix the samples and then incubated 30 minutes at 37°C. After incubation period the plate was read at 630 nm.

**Measurement of Sirtuin 1 levels:** SIRT1 levels were quantified using SIRT1 Sandwich Enzyme Immunoassay from Mybiosource (Cat # MBS2023445). This kit includes a 96-well strip plate pre-coated with an antibody specific to SIRT1. 100 µl of standards or samples were added to the plate wells with a biotin-conjugated antibody specific to SIRT1 and incubated for 2 hours at 37°C. 100 µl of Detection Reagent A working solution was added to each well and the plate covered and incubated for 1 hour at 37°C. After the incubation period the plate was washed three times with 350 µl of wash solution for each well using multi-channel pipette. The plate was allowed to sit for 2 minutes and excess liquid was removed by pressing the plate onto absorbent paper towels. 100 µl of Detection Reagent B working solution was then added to each well and the covered plate was incubated for 30 minutes at 37°C. The plate was then washed with 350 µl of wash solution five times. 90 µl of Tetramethylbenzidine (TMB) Substrate Solution was then added to each well (samples turned blue) and the covered well plate was incubated for 15 minutes at 37°C. The plate was protected from light. 50µL of Stop Solution was added to each well to stop the reaction and the plate was placed on a shaker to mix the samples well. A microplate reader was used to measure the OD readings of the standards and samples at 450nm.
**Measurement of AMPK-P levels:** The AMPK-P levels in the samples were quantified using an AMPKα pT172 ELISA Kit (Cat # ab154468). This kit contains a 96 well plate which is pre-coated with a specific mouse monoclonal antibody specific for AMPKα pT172. 25 µl of standards (Hek293T cells) or samples were added to the wells along with the rabbit monoclonal primary detector antibody and the plate was incubated for three hours at room temperature. The plate was then washed with 300 µl of 1X wash buffer to eliminate any unbound standard or samples in the plate. After washing and drying of the plate, 50 µl of horseradish peroxidase (HRP)- conjugated secondary detector antibody (HRP Label) specific for the primary detector antibody was added to wells. After incubation, the well plate was washed again three times using 300 µl of 1X wash buffer. Then 100 µl of a Tetramethylbenzidine (TMB) substrate solution was added to the wells and the wells changed in color depending on the amount of AMPKα pT172 bound present in the samples. The OD of the samples was then measured at 600 nm and used to calculate the amount of each AMPK-P in each sample. A standard curve was created and the unknown samples were extrapolated this standard curve.

**Statistical Analysis:** For the statistical analysis of experiments, we used Graphpad Instat Software (Graphpad, La Jolla, CA, USA). Statistical comparisons between two-groups was performed using the unpaired Student’s t-test. All data are mean±SD represented by the error bars. Means were considered significantly different when p<0.05.
Results

**THS exposure leads to increased oxidative stress in the liver**

Previously, we showed that 30% of the mice exposed to THS toxins develop a fatty liver [32]. We hypothesize that exposure to THS toxins leads to lipid accumulation in the liver of THS-exposed mice by increasing oxidative stress. Under normal conditions, the hepatocytes balance oxidative stress with the help of two important antioxidant enzymes, catalase and glutathione peroxidase (GPx). These enzymes regulate the levels of hydrogen peroxide (H$_2$O$_2$) in the liver. Catalase converts H$_2$O$_2$ into water and oxygen whereas GPx converts it into water only (Figure 2.1A). THS-exposed mice have higher superoxide dismutase (SOD) activity and as a result they also have higher levels of H$_2$O$_2$ in the liver than do the controls (Figure 2.1B-C). Because there is no significant increase in catalase or GPx activity, H$_2$O$_2$ is not properly been processed and the levels of reactive oxygen species (ROS) remain high in the liver (Figure 2.1 D-E). To further support these results, we measured the NADP$^+$/NADPH ratio, which is an indicator of the cell reducing potential, because GPx activity is coupled to the reduction of NADPH to NADP$^+$. The NADP$^+$/NADPH ratio is significantly lower in THS-exposed mice than in control mouse livers (Figure 2.1F), showing that the reduction potential of these animals is decreased and might result in their inability to reduce the oxidative stress levels induced by the THS toxins.

**THS-induced ROS lead to molecular and cellular damage in the liver**
High levels of H$_2$O$_2$ in cells can lead to the production of hydroxyl radicals via the Fenton reaction in the presence of ferrous iron ion (Fe$^{2+}$) (Figure 2.2A). These radicals can damage lipids and proteins, and lead to the formation of DNA adducts. We found that in the liver of THS-exposed mice there are significantly higher levels of lipid peroxidation, protein nitrosylation and DNA damage (Figure 2.2B-D and 2.S1). We also investigated whether THS toxins result in functional damage in the liver by measuring ATP, aspartate aminotransferase (AST) and urea levels. ATP is a marker for mitochondria function whereas AST and urea are markers of liver health. THS-exposed mice produced lower ATP levels than the controls (Figure 2.2E), suggesting that the ability of the mitochondria in THS-exposed mice to synthesize cellular energy is decreased. High levels of AST suggest liver damage whereas high levels of urea suggesting increase in amino acid metabolism (Figure 2.2F,G). Together, these results show that THS toxins result in damage of liver function.

**Oxidative stress mediated damage is improved by treating the mice with antioxidants**

To investigate whether the THS-induced oxidative stress in the liver can be reversed, we treated the THS-exposed mice with the antioxidants N-acetylcysteine (NAC) and α-tocopherol (α–TOC) for five months. These two antioxidants have been used previously to treat oxidative stress in different tissues [46-48]. Treatment with these antioxidants for five months while the mice were being exposed to THS, leads to a decrease in oxidative stress. These mice have SOD activity and H$_2$O$_2$ levels similar to
those of the non-exposed mice treated with antioxidants but significantly lower than THS-exposed mice without treatment with antioxidants (Figure 2.3A,B). Although no significant changes were observed in the activity of catalase (Figure 2.3C), the THS-exposed mice treated with antioxidants have higher GPx activity than THS-exposed non-treated mice (Figure 2.3D). The same occurred for the NADP⁺/NADPH ratio (Figure 2.3E). Furthermore, NAC and α-TOC lowered the total hepatic lipid weight in the THS-exposed mice (Figure 2.3F).

Treatment with antioxidant agents also decreased the oxidative-stress-mediated molecular damage in THS-exposed mice. These mice have lower lipid peroxidation (Figure 2.4A) and lower protein damage (Figure 2.4B) in THS exposed mice. However, no significant improvement was observed in DNA damage (Figure 2.4C). We also investigated whether antioxidant treatment improved functional damage by measuring ATP, AST and urea. AST is responsible for the conversion of aspartate to α-ketoglutarate to oxaloacetate and glutamate, which is fundamental for the function of the liver. Urea is a by-product of amino acid metabolism that occurs in the liver. Antioxidant treatment did not improve these parameters (Figure 2.4D-F).

**Treatment of THS-exposed mice with APAP increases oxidative-stress-mediated damage**

Because N-acetyl-p-aminophenol (APAP/Tylenol) is a common drug taken by many people, especially by children, and it is known to damage the liver if taken in high doses [49], we investigated whether giving THS-exposed mice Tylenol would further increase
oxidative stress in the liver. We used a dose of APAP/Tylenol that has been previously shown to induce chemical damage in the liver without killing the animals [49-51]. Based on these studies, we treated the THS-exposed mice for eight weeks with APAP/Tylenol. THS-exposed mice treated with APAP/Tylenol have higher SOD activity and higher H$_2$O$_2$ levels than THS-exposed non-treated mice (Figure 2.5A and B). Moreover, the levels of catalase activity were significantly decreased when compared with THS-exposed non-APAP treated animals (Figure 2.5C). We also observed that although GPx activity was not changed (Figure 2.5D), the NADP$^+/$/NADPH ratio was significantly decreased (Figure 2.5E), showing that the reducing potential in the THS-exposed APAP-treated mice was low. Furthermore, APAP/Tylenol increased the total hepatic lipid weight in the THS-exposed mice (Figure 2.5F). This results in further damage to the ability of the liver to handle the oxidative stress damage induced by THS.

We also quantified the oxidative stress-induced molecular damage and observed that THS exposure in conjunction with APAP/Tylenol treatment results in much higher levels of lipid peroxidation (Figure 2.6A), higher protein nitrosylation (Figure 2.6B) and higher DNA damage (Figure 2.6C). In addition, we also measured the ATP, AST and urea levels. High levels of AST and urea are associated with abnormal liver function [52-53]. APAP treatment results in lower ATP levels in THS-exposed mice suggesting that these mice have dysfunctional mitochondria (Figure 2.6D). APAP/Tylenol treatment results in higher AST levels in these animals suggesting that when THS exposure and APAP/Tylenol treatment are combined, further functional damage to the liver occurs (Figure 2.6E). However, APAP/Tylenol treatment of the THS-exposed mice did not have
increased urea levels when compared to exposure only to THS (Figure 2.6F).

**THS toxins inhibit the SIRT1/AMPK/SREBP1c signaling pathway in the liver.**

To investigate the cellular and molecular mechanisms of THS-induced lipid metabolism, we performed genomic analysis using both DNA microarray and RNA-Seq and found that Sirtuin 1 (SIRT1), a key regulator of lipid metabolism, is down regulated in THS-exposed mice. SIRT1 modulates the activity of other metabolic energy regulators such as 5' AMP-activated protein kinase (AMPK). AMPK is an energy sensor within the cell that responds to energy depletion (decrease in ATP production) or any other cellular damage insult that result in alterations to cellular energy. Together, SIRT1 and AMPK regulate lipid metabolism by preventing the synthesis of lipids and by stimulating fatty acid oxidation [54-68]. It has been shown using SIRT1 transgenic mice showed SIRT1 is required for activation of that AMPK and improvement of mitochondrial function [67]. It has also been shown AMPK phosphorylates and inhibits SREBP1c in diet induced insulin resistant mice [68]. Taken together studies suggest that SIRT1 and AMPK both work to prevent abnormal lipid metabolism by targeting and inhibiting SREBP1c a key lipogenic enzyme in the liver. Therefore, we investigated the SIRT1/AMPK/SREBP1c pathway and whether the THS-induced hepatic steatosis is a result of alterations in this pathway. When compared to the control mice the THS-exposed animals have lower levels of SIRT1 and of phosphorylated AMPK, the active form of this enzyme (Figure 2.7A,B). Because activated AMPK is decreased in THS-exposed mice we tested the levels of SREBP1c the levels of SREBP1c. We found that in THS-exposed mice
SREBP1c is elevated, leading to higher levels of lipids in the liver (Figure 2.7C). These findings suggest that THS exposure stimulate hepatic steatosis by altering the SIRT1/AMPK/SREBP1c pathway.
Discussion

We have previously shown that THS exposure results in oxidative stress mediated damage of multiple organs in mice including steatosis of the liver [32]. Here we show that the lipid accumulation is correlated with increase in the levels of oxidative stress, primarily of ROS, and inhibition of the SIRT1/AMPK/SREBP1c pathway (Figure 8A). Modulating the levels of ROS with antioxidants resulted in reduction of the THS-induced hepatic steatosis whereas treatment with APAP resulted in augmentation of the THS-induced the lipid accumulation (Figure 8B).

We showed that, in the THS-exposed mice, the oxidative stress did not result in organ failure nor did the mice die. Data from a cytokine array performed on the serum of these mice showed that there is a small increase in pro-inflammatory cytokines and chemokines, indicating that THS-exposed mice have very low levels of inflammation [32] but we do not think that the low levels of inflammation have an effect on development of steatosis. For example, TNF-α and IL-1β, cytokines that have been associated with hepatic steatosis, were not altered in the THS-exposed mice [69-71]. However, we did see significant changes in SIRT1, AMPK and SREBP1c, which are known to be critically involved in lipid metabolism. Therefore, we focused our studies on the SIRT1/AMPK/SREPB1c pathway to determine if oxidative stress altered this pathway and resulted in THS-induced hepatic steatosis.

The oxidative stress mediated damage to the hepatic lipids, proteins, and DNA have a detrimental effect for lipid metabolism [72-74]. The liver is one of the major metabolic organs where glucose is metabolized, modified and stored. Perturbations in the
metabolism and storage of glucose as well as insulin resistance in the liver have been
linked to the development of hepatic steatosis [74-76]. It is known that lipid peroxidation
results in a decrease in the uptake of glucose in cortical rat neurons, which in the short
term leads to abnormal glucose metabolism but over time it may lead to cell death [77].
Lipid peroxidation can also lead to conformational changes in the lipid bilayer and these
changes can potentially alter the localization and stability of many receptors involved in
lipid metabolism [78].

THS-exposed mice have high levels of protein damage, which affect the
expression, and levels of proteins involved in the regulation of lipid metabolism in the
liver. SIRT1 is a key regulator of lipid metabolism and it is known to work together with
AMPK to prevent lipid accumulation in the liver by inhibiting SREBP1c and increasing
fatty acid metabolism [54-68]. In this study we show that THS toxins alter the
SIRT1/AMPK/SREBP1c signaling axis by decreasing SIRT1 levels which lead to
decrease in phosphorylated/activated AMPK resulting in increase of SREBP1c and
increase in lipid synthesis, with subsequent development of hepatic steatosis (Figure 8A).
We also show that the liver specific damage we observed in the THS-exposed mice is
mediated by oxidative stress triggered by the THS toxins. Increasing the oxidative stress
in the liver of THS-exposed mice with APAP/Tylenol worsened lipid accumulation
whereas when the mice were exposed to THS and treated with antioxidants there was a
decrease in lipid accumulation.

APAP/Tylenol is commonly used to treat headaches and fever and when taken for
prolonged periods of time or higher dose it is known to cause liver damage. This can
occur by depletion of glutathione (GSH) and by increasing mitochondria damage [79-81]. When we treated THS-exposed mice with APAP, we observed that these mice have higher liver damage than mice only exposed to THS. These mice also have lower levels of ATP, which suggest impaired mitochondria function. AST levels were also elevated suggesting abnormality in amino acid metabolism in the liver.

To decrease oxidative stress we treated THS-exposed mice with NAC and α-TOC, which have been commonly used in humans and rodents for that purpose [82-84]. NAC is derivative of the amino acid L-cysteine and it is a direct precursor of glutathione consequently it has direct and indirect antioxidant properties [83]. Its free thiol group can interact with the electrophilic groups found in reactive oxygen species (ROS) agents. NAC can behave as an indirect antioxidant in the glutathione synthesis pathway, an antioxidant enzyme that decreases cellular oxidative stress [83]. α-TOC is an important lipid-soluble antioxidant that reacts with lipid radicals and consequently less lipid peroxidation occurs preventing damage to the plasma and mitochondrial membranes [84].

In conclusion, these studies provide insight into the effects of THS toxins on key molecules in lipid metabolism such as the SIRT1/AMPK/SIRT1 signaling pathway. We show that by altering the oxidative stress levels using APAP/tylenol or the antioxidant agents NAC and α-TOC, we alter the THS-induced damage, which indicates that the THS-induced damage is mediated by oxidative stress. Based on our results, it is clear that THS toxins have the ability to alter fundamental metabolic processes such as lipid metabolism resulting in steatosis making THS a new risk factor for hepatic steatosis.
References


Figure 2.1. THS exposure results in oxidative stress in the liver. (A) Schematic depicting basic oxidative stress response in cells. (B) THS-exposed mice have increased SOD enzymatic activity compared to control mice. (C) THS-exposed mice show increased hydrogen peroxide in the liver compared to control mice. (D) THS-exposed mice do not show significant decrease in catalase enzymatic activity compared to control. (E) THS-exposed mice also do not show a significant difference in GPx activity compared to control mice. All data are Mean ± SD * p< 0.05 NS = Not statistically significant. n=6. P values were adjusted for the number of times each test was run.
Figure 2.1
Figure 2.2. THS exposure increase oxidative stress mediated damage. (A) Model of oxidative stress induced mediated damage in cells. (B) THS-exposed mice have higher lipid peroxidation in the liver compared to control mice (C) THS-exposed mice show higher protein nitrosylation in the liver compared to control mice (D) THS-exposed mice exhibit higher DNA damage in the liver compared to control. (E) THS toxins also decrease ATP levels, (F) increase AST levels and urea in THS exposed mice (G). All data are Mean ± SD *= p< 0.05 NS= Not statistically significant. n= 8 for B,C, and D. n=6 for E-G. P values were adjusted for the number of times each test was run.
Figure 2.2

A. Fenton Reaction Diagram

B. Lipid Peroxidation

C. Protein Nitrosylation

D. DNA Damage

E. ATP Liver

F. AST Activity

G. Urea Liver
Figure 2.3. Antioxidant treatment leads to improvement oxidative stress in the liver of THS-exposed mice. (A) THS-exposed mice treated with the NAC+ α-TOC have significantly reduced SOD activity and (B) low levels of H$_2$O$_2$. (C) NAC+α-TOC treatment has no significant change in the enzymatic activity of catalase in THS-exposed mice (D) NAC+ α-TOC treatment also did not led to a significant change in the enzymatic activity of GPx but it resulted in (E) significant increase of NADP+/NADPH ratio in THS exposed mice. * = p< 0.05 NS= Not statistically significant n= 9 for A and E n= 8 for B,C, and D. P values were adjusted for the number of times each test was run.
Figure 2.3
Figure 2.4. Oxidative stress mediated damage is improved by treating the mice with antioxidants (A) THS-exposed mice treated with NAC+ α-TOC show lower lipid peroxidation (B) THS-exposed mice treated with NAC+ α-TOC also show lower protein nitrosylation but (C) no significant difference in DNA damage. (D) THS-exposed mice treated with NAC+ α-TOC do not have a significant increase in ATP levels, or (E) AST levels or (F) urea levels. All data are Mean ± SD *= p< 0.05, NS= Not statistically significant. n=8 for A n=7 for B n=9 for C,D E,F. P values were adjusted for the number of times each test was run.
Figure 2.4
Figure 2.5. APAP treatment results in increase of oxidative stress in THS-exposed mice. (A) APAP treatment results in higher SOD activity in THS-exposed mice. (B) APAP treatment also results in higher levels of hydrogen peroxide in THS-exposed mice. (C) APAP treatment also resulted in a decrease in catalase activity but no significant change in (D) GPx activity. (E) APAP treatment also did not had a significant impact in the NADP⁺/NAPPH ratio. All data are Mean ± SD *= p< 0.05 NS= Not statistically significant n= 9 for A and E n= 8 for B,C, and D. P values were adjusted for the number of times each test was run.
Figure 2.5
Figure 2.6. THS-exposed mice treatment with APAP increases oxidative stress-mediated damage (A) APAP treatment results in higher lipid peroxidation, (B) higher protein nitrosylation, and (C) higher DNA damage in the liver of THS exposed mice. (D) THS-exposed mice treated with APAP also have lower ATP levels; (E) higher AST levels but (F) lower urea levels than THS-exposed mice. All data are Mean ± SD * = p < 0.05, NS = Not statistically significant. n=8 for A n=7 for B n=9 for C,D E,F. P values were adjusted for the number of times each test was run.
Figure 2.6
Figure 2.7. THS toxins alter SIRT1/AMPK/ SREBP1c signaling in the liver.

(A) Antioxidant treatment and THS exposure results significant lower total lipid hepatic weight in THS-exposed mice. (B) APAP treatment in conjunction of THS exposure also results in higher total lipid hepatic weight. (C) THS-exposed mice have less SIRT1 levels than controls, (D) lower AMPK-P levels and (E) higher levels of SREBP1c than control mice. *= p< 0.05, ** p<0.01 NS= Not statistically significant. n=6 P values were adjusted for the number of times each test was run. “Fold change” on western blot quantification graph indicate fold change to control.
Figure 2.7
Figure 2.8. Proposed working model of THS-induced hepatic steatosis. A schematic representation of THS-induced hepatic steatosis in mice depicting how THS toxins increases oxidative stress in the liver, which results in decrease of SIRT1 levels, a key regulator oxidative stress and regulate lipid metabolism. The toxins also result in a decrease in the levels of active AMPK and increase in SREBP1c levels, key regulator of de novo lipid metabolism. As a result THS exposure induces fat accumulation in the liver which results in hepatic steatosis.
Figure 2.8
**Figure S1. Immunolabeling for lipid peroxidation.** Representative images of liver tissue staining for 4-HNE lipid peroxidation adducts. Livers of control mice have less lipid peroxidation than control livers.
CHAPTER 3:

The role of fatty acid metabolism and apolipoproteins in THS-induced hepatic steatosis in mice
Abstract

Background: Hepatic steatosis results from the increase of accumulation of lipids in the liver, decrease of beta fatty acid oxidation and/or decrease in the export to peripheral tissue by apolipoproteins. Previously, we showed that THS toxins result in hepatic steatosis in mice.

Objective: The goal of this paper was two-fold: (1) To determine whether THS toxins alter key molecules involved in beta fatty acid metabolism and (2) to determine whether the levels of apolipoprotein B is decreased in THS-exposed mice leading to decrease export of lipid from the liver.

Methodology: Mice were exposed to THS toxins for 6 months before performing the studies shown here. THS-exposed mice were also placed on western diet for five months or treated with AICAR to determine how THS-toxins affect the lipid metabolism of these animals.

Results: THS-exposed mice do not show significant difference in the levels of key fatty acid metabolism enzymes (CPT1, ACC, IDH2 and LCAD) compared to the control, suggesting THS toxins do not decrease the levels of these enzymes. THS-exposed mice have lower levels of SIRT3 and ATP. These mice also have lower IDH2 activity. THS-exposed mice also have lower levels of apolipoprotein B compared to control, suggesting the excess fatty acids, which are converted to TG in the liver, are not being transported to peripheral tissue for usage or storage.

Conclusion: These results suggest that even thought THS toxins do not alter the levels of fatty acid metabolism enzymes, exposure result in lower levels of SIRT3 and lower IDH2
activity resulting in lower production of ATP in THS-exposed mice. THS toxins exposure also decrease of transport of lipids out of the liver by decreasing the levels of apolipoprotein B. Consequently, THS-exposed mice have an increase in lipid accumulation in the liver resulting in hepatic steatosis.
Introduction

The liver is one of the largest and most metabolically active organs in the body. In addition to performing a myriad of metabolic functions that regulate energy levels, the liver is a major site for protein synthesis and is responsible for the production of lipids to be delivered to other tissues [1-2]. Under normal conditions, the liver is constantly regulating synthesis and breakdown of lipids to meet physiological metabolic demands. In the liver, AMPK and SREBP1c regulate the synthesis of lipids in the liver. When there is an excess of fatty acids in the liver, SREBP1c is activated and activates acetyl-coA carboxylase (ACC), which leads to the activation of malonyl CoA, resulting in the synthesis of triglycerides (TGs) [4-8]. If not secreted into circulation, these TGs accumulate in the liver and can result in hepatic steatosis. Conversely, the liver can increase breakdown of fatty acids via beta-oxidation to decrease lipid accumulation by decreasing the activation of SREBP1c and increasing the activation of key mitochondrial enzymes that participate in the oxidation of fatty acids, such as carnitine palmitoyltransferase I (CPT1), long-chain acyl-CoA dehydrogenases (LCAD), and isocitrate dehydrogenase 2 (IDH2). These enzymes catalyze fatty acid breakdown starting by transporting the activated fatty acids into the mitochondria by CPT1. LCAD then further processes the fatty acids that enter the tricarboxylic acid (TCA) cycle in the form of acetyl-CoA. The processing of the fatty acids continues via the participation of IDH2, which allows the fatty acids to be converted into high-energy molecules that can be used for the synthesis of ATP during oxidative phosphorylation. Therefore, low ATP production can be a result of a decrease in levels or function of LCAD or IDH2 [9-14].
Perturbation of this catabolic pathway can result in abnormal fatty acid metabolism, which can consequently lead to the accumulation of lipids in the hepatocytes, resulting in hepatic steatosis.

Another path by which the hepatocytes can further control lipid accumulation is by transporting excess lipids out of the liver by increasing very low density lipoprotein (VLDL) assembly and secretion. A decrease in VLDL assembly and secretion has been associated with hepatic steatosis [15-17]. VLDL particles, which are synthesized in the liver, are responsible for delivering TGs to the different cells of the body. VLDL particles are composed of TG, cholesterol and apolipoprotein (apoB). In the liver, VLDL synthesis is greatly affected by the levels of microsomal triglyceride transfer protein (MTTP) and apoB [18-26]. ApoB in particular has been associated with non-alcoholic fatty liver (NAFLD). Past studies have shown that insulin has the ability to inhibit the expression of apoB, whereas fatty acids induce its expression [20-21]. Also, patients with NAFLD have reduced expression of apoB, suggesting that their liver is unable to transport TG into peripheral tissue, resulting in accumulation of lipids in the hepatocytes [18]. The function of apoB is affected by MTTP proteins which allow apoB to be released from the ER and start participating in the organization of the VLDL particles. Low levels of MTTP have also been associated with the degradation of apoB and the decrease of VLDL particles [23-26]. Therefore, altering the level of MTTP and apoB in the liver affects the transport of lipids out of the liver. Consequently, lipid accumulation results in hepatic steatosis.

The ability of the liver to process fatty acids and transport lipids to other tissues is
affected by toxins, diet and drugs [27-34]. Previously, we have shown that exposure to THS toxins results in lipid accumulation in hepatocytes of exposed mice. Recently, we have shown that these mice also have high levels of oxidative stress that lead to lung and liver damage as well as abnormal cognitive behavior [3]. We also showed that THS toxins result in altering the SIRT1/AMPK/SREPB1c pathway [34]. Modulating the levels of oxidative stress with APAP (Tylenol) and antioxidants resulted in worsening and improvement of hepatic steatosis, respectively, in mice exposed to THS toxins. These findings suggested that THS toxins result in hepatic damage by altering the oxidative stress levels and increasing lipid synthesis. The goal of this study is to determine whether THS also affects fatty acid metabolism and lipid transport. Our results show that THS toxins decrease the activity of IDH2 leading to decrease in lipid breakdown and ATP production and that they also decrease the levels of MTTP and apoB, suggesting alterations in the lipid transport system. Our findings show that THS toxins affect the metabolism and transport of lipids in mice while highlighting the danger of THS toxins for liver health.
Materials and methods

Animals: All male C57BL/6 mice used for these experimental procedures were housed under the following environmental conditions: 12-hour light/dark cycle in convectional cages with *ad libitum*. The regular standard chow diet was composed of 58% carbohydrates, 28.5% protein, 13.5% fat and water.

Ethics Statement: The experiments were done according to animal protocols that were approved by the University of California, Riverside, Institutional Animal Care and Use Committee (UCR-IACUC). The mice used for these studies were euthanized by carbon dioxide (CO$_2$) inhalation, which is the most common method of euthanasia used by NIH. The amount and length of CO$_2$ exposure were approved by UCR-IACUC.

THS exposure method: The THS exposure method used for these studies has been previously described in detail (3). C57BL 6J mice were housed in THS-exposed material to study the effects of toxins in liver health for a period of 24 weeks. THS exposure of the material (10 g curtain composed of cotton, 10 g upholstery composed of cotton and 10 g carpet composed mainly of fiber) was done using a Teague smoking apparatus. The machine was set to smoke two packs of 3R4F research cigarettes five days a week to model a regular smoker (cigarette/15 min). The total particulate matter (TPM) was set to 30µg/m$^3$, which is the TPM found in the homes of smokers. The mice were housed in the TSH-exposed material for a week and the material was exposed for four weeks to allow the toxins to age. After four weeks the material was discarded. The control C57BL 6J
mice were not exposed to THS toxins and were housed in a different room than the THS-exposed mice.

**Western Diet treatment:** After weaning, normal C57 BL/6J male mice and Third-Hand Smoke (THS)-exposed C57 BL/6J male mice were placed on a western chow diet for duration of 6 months. The western diet was purchased from Harlan laboratories (Cat# TD88137) and was composed of 21% anhydrous milk fat (butterfat), 34% sucrose, and 0.2% cholesterol.

**AICAR treatment:** AICAR was purchased from Toronto Research Chemicals (Cat# A440500) and was dissolved in phosphate buffered saline (PBS). THS-exposed or control mice were given an intraperitoneal injection (I.P) of either AICAR (250 mg/kg for 5 days/week) or a comparable volume of PBS for eight weeks.

**Tissue Extracts:** After six months of THS exposure, the livers of THS-exposed male mice were extracted and immediately frozen and stored at -80°C. Protein extracts were made from portions of the frozen tissue unless otherwise specified by the kits’ instruction manuals, and were used for ELISA analysis. Liver tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA), centrifuged, and the supernatant collected unless otherwise specified by the kits’ instruction manuals.
**Blood Extracts:** Blood was extracted directly from the heart and allowed to coagulate for 20-30 min. The samples were then centrifuged at 2000 RPM to induce phase separation between plasma proteins and serum. The serum was then collected from the blood samples. The serum was used immediately for assays or immediately frozen and stored at -80° C.

**Total lipid:** Lipid content in the liver was determined using the Folch method (7). Tissue was homogenized with chloroform/methanol (2/1) to a final volume 20 times the volume of the tissue sample and agitated for 15-20 min. The homogenate was centrifuged to recover the liquid phase. The solvent was washed with 0.2 volume of 0.9% NaCl solution. After vortexing, the mixture was centrifuged at 2000 RPM to separate the two phases. The upper phase was removed and the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator. To determine the total amount lipids in the sample the weight of the vial was subtracted from the weight of the vial plus lipids.

**Measurement of fatty acids (FA) levels:** Fatty acids were quantified using Abcam kits (Cat #ab65341). In this assay, FAs are converted to their CoA derivatives and subsequently oxidized, leading to formation of color/fluorescence. Fatty acid levels were quantified using the absorbance values obtained from the liver tissue homogenates samples at 550 nm.

**Measurement of triglyceride (TG) levels:** The TG contents were measured using Cayman Triglyceride Colorimetric Assay Kit (Cat# 10010303). The Triglyceride
Colorimetric Assay uses the enzymatic hydrolysis of triglycerides by lipase to produce glycerol and free fatty acids. The absorbance of standards and samples were read at 550 nm. Absorbance values were used to generate a standard curve. The levels of TG were interpolated from this standard curve.

**Measurement of IDH2 levels:** The IDH2 levels in liver of THS-exposed mice were quantified using IDH2 ELISA kit from Mybiosource (Cat# MBS074031). IDH2 levels were quantified by incubation of tissue homogenate in an anti-IDH2 antibody coated plate, followed by an HRP conjugated secondary antibody. The optical density (O.D.) of the samples was then read at 450 nm. The values were used to quantify the levels of IDH2 in each sample.

**Measurement of LCAD levels:** The LCAD levels in THS-exposed mice were quantified using the ELISA kit from Mybiosource (Cat# MBS931062). LCAD levels were quantified by incubation of tissue homogenate in an anti-LCAD antibody coated plate, followed by an HRP conjugated secondary antibody. The optical density O.D. of the plate was then read at 450 nm. The values were used to quantify the levels of LCAD in the liver.

**Measurement of CPT1 levels:** The CPT1 levels of THS-exposed mice were quantified using CPT1 ELISA kit from Mybiosource (cat# MBS7252964). CPT1 levels were quantified by incubation of tissue homogenate in an anti-CPT1C antibody coated plate followed by detection by substrate solution provided in the kit. The O.D. of the plate was then read at 450 nm. The values were used to quantify the levels of CPT1 in each sample.
**Measurement of ACC levels:** ACC levels were determined using an ELISA kit from Mybiosource (Cat# MBS2606160). ACC levels were quantified by incubation of liver tissue homogenate in an anti-ACC antibody coated plate, by detection by substrate solution provided in the kit. The O.D. of each well was measured at 450 nm. The resulting O.D. values were used to calculate the ACC levels in the liver.

**Measurement of apoB levels:** The apoB levels in liver of THS-exposed mice were quantified using apoB ELISA kit from Neoscientific (Cat# MA0520). APOB levels were quantified by incubation of tissue homogenate in an anti-apoB antibody coated plate. The O.D. of the plate was then read at 450 nm. The values were used to quantify the standard curve, which was used to calculate the levels of apoB in the liver samples.

**Measurement of MTTP levels:** MTTP levels in the livers of THS-exposed mice were quantified using MTTP ELISA kit from Mybiosource (Cat# MBS9329329). MTTP levels were quantified by incubation of tissue homogenate in an anti-MTTP antibody coated plate, followed by an HRP conjugated secondary antibody. The optical density O.D. of the plate was then read at 450 nm. The values were used to generate a standard curve and to quantify the levels of MTTP in the liver.

**Measurement of VLDL levels:** VLDL levels in the livers of THS-exposed mice were quantified using VLDL ELISA kit from Mybiosource (Cat# MBS706037). VLDL levels were quantified by incubation of tissue homogenate in an anti-VLDL antibody coated plate, followed by an HRP conjugated secondary antibody. A standard curve was
generated and used to quantify VLDL levels in liver tissue of THS-exposed mice and control mice.

**Statistical Analysis:** For the statistical analysis of experiments mentioned above, we used Graphpad Instat Software (Graphpad, La Jolla, CA, USA). Statistical comparisons between two-groups was performed using the unpaired Student’s t-test. All data are mean±SD represented by the error bars. Means among samples were considered significantly different when p<0.05.
Results

Hepatic steatosis can result from an increase of de novo lipid synthesis, decrease of breakdown of fatty acids, or decrease of the transport of lipids out of the liver. Previously, we showed that THS toxins increase oxidative stress in the liver of mice resulting in hepatic steatosis [34]. In this study we investigated whether THS-exposure changes the levels and/or activity of key enzymes involved in the breakdown of fatty acids and in the transport of lipids out of the liver.

**THS-exposure affects FA, TG and total lipid levels in the liver**

Quantification of fatty acids, TG and lipids in THS-exposed mice showed higher levels of all three in the liver of THS-exposed mice (Figure 3.1A-C). Because western diet (WD), which is rich in fat (see Materials and Methods section) and has been shown to decrease the activation of AMPK, an enzyme that regulates the breakdown of fatty acids in the liver [35], we tested whether WD augments the effects caused by THS-exposure. Mice were placed at weaning (3 weeks of age) on WD in conjunction with THS exposure for 5 months. At the end of this period we analyzed again for FA, TG and total hepatic lipids and found that none of these parameters were significantly higher when compared to the mice on the chow diet and exposed to THS (Figure 3.2A-C). However, there was a significant difference in the levels compared to those of mice feed WD only (Figure 3.2A-C).

To investigate whether an stimulator of AMPK phosphorylation, 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) [35], resulted in
improved fatty acid metabolism in the THS-exposed mice, we treated mice exposed to THS for 6 months with AICAR at 250 mg/kg for 5 days/week for eight weeks via an intraperitoneal injection and treated the controls in a similar way with comparable volume of PBS. Indeed, we found that AICAR was able to reverse the adverse effects of THS exposure in lipid metabolism. The levels of fatty acids, TG and total lipids were returned to normal levels even in the presence of THS exposure (Figure 3.3 A-C).

**Effects of THS-exposure on the levels/activity of enzymes involved in fatty acid metabolism.**

Based on the results presented above we examined whether THS-exposure affects the levels of key enzymes that play a role in the breakdown of fatty acids in the liver. For these studies we measured the levels of Acetyl-CoA carboxylase (ACC), carnitine palmitoyltransferase I (CPT1), long-chain acyl-CoA dehydrogenases (LCAD), and isocitrate dehydrogenase 2 (IDH2) after six months of exposure. Except for IDH2, that we found to have a tendency to be decreased, the other three enzymes did not show levels different from the control and the THS-exposed mice (Figure 3.4A-D). Because, we observed that IDH2 levels were consistently lower in THS-exposed mice although be it not quite significantly lower, we tested for activity of IDH2 and found that its activity is significantly decreased in THS-exposed mice (Figure 3.5A).

Because it is known that SIRT3 plays a key role in fatty acid metabolism and that it modulates the activity of IDH2, we examined the levels of SIRT3 in THS-exposed mice. We found lower levels of SIRT3 in the liver of THS-exposed mice when compared
to the control suggesting that these mice might have lower fatty acid breakdown than the controls (Figure 3.5B). Because IDH2 is an important enzyme in the TCA cycle, which is key for the production of the reduction potential in the cell (NADPH an FADH2), which then provides the electrons to the oxidative phosphorylation chain to produce ATP, we measured the level of ATP to determine whether it was affected by THS exposure. We found that ATP levels were significantly reduced in the liver of THS-exposed mice (Figure 3.5C). Taken together these results suggest that the capacity of lipids to undergo β-oxidation in the liver is reduced by THS- exposure, which very likely leads to the accumulation of lipids in the hepatocytes.

**THS exposure results in a decrease of VLDL assembly in the liver.**

To determine whether THS-exposure affects lipid transport out of the liver, we investigated whether THS toxins alter the level of proteins involved in lipid transport out of the liver. Both apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTTP) have been associated with the transport of lipids out of the liver into peripheral tissues such as to the adipose tissue. We focused on apoB and MTTP because these proteins play a key role in VLDL assembly, a particle type that is critical in transport of lipids out of the liver [23-26]. THS-exposed mice showed lower levels of apoB (Figure 3.6A) and MTTP (Figure 3.6B) in the liver compared to the controls. In addition, we also found that THS-exposed mice had lower VLDL levels than controls (Figure 3.6C). These findings indicate that THS toxins alter processes involved in lipid transport out of the liver through decreased levels of VHDL particle formation and consequently this contributes to lipid accumulation in the liver of THS-exposed mice.
Discussion

We have previously shown that THS exposure results hepatic steatosis [3]. More recently we showed that, THS-exposed mice have high levels of hepatic oxidative stress leading to liver damage. We also showed that the lipid accumulation observed in THS-exposed mice is due to increase in the levels of oxidative stress, primarily of ROS, and inhibition of the SIRT1/AMPK/SREBP1c pathway [34]. The goal of this study is to determine whether THS toxins affect fatty acid metabolism and lipid transport in THS-induced hepatic steatosis.

In this study, we show that THS-exposed mice are unable to metabolize and transport lipids efficiently compared to the control mice. These mice have higher lipids, fatty acids and TG suggesting these animals do not have the metabolic efficiency exhibited by the control mice. WD diet has been associated with increase lipid accumulation in the liver whereas AICAR treatment has been associated with a decrease in lipid accumulation [35-40]. Western diet has been shown to increase oxidative stress and increase fat accumulation in the liver by decreasing fatty acid metabolism resulting in hepatic steatosis. THS-exposed mice placed on WD did not have a worse lipid profile than the THS-exposed mice placed on chow diet. However, AICAR treatment led to improved lipid metabolism in THS-exposed mice. THS exposed mice treated with AICAR had lower FA, TG and hepatic lipids. These finding suggest AICAR is able to reverse the lipid abnormalities induced by THS toxins but WD is unable to further worsen the lipid abnormalities triggered by THS toxins.
Fatty acid metabolism plays a key role in the development of hepatic steatosis [33]. In our previously we showed THS-toxins increase the oxidative stress levels and lower SIRT1 levels (34). Consequently, THS-exposed mice have higher lipid synthesis than the control. In this study we focused on SIRT3 because it is a key regulator of fatty acid breakdown. SIRT3 plays a key role in lipid homeostasis in the liver and by increasing fatty acid metabolism in the mitochondria. This sirtuin also regulates fatty acid metabolism by enhancing the activity of IDH2, which is involved in processing the fatty acids via the TCA cycle in the liver [11-12]. THS-exposed mice show lower levels of SIRT3 as well as lower activity of IDH2 suggesting than THS toxins alter lipid metabolism by also targeting the breakdown of fatty acids in the mitochondria. Consequently, THS-exposed mice do not have the same metabolic efficiency as the controls; they have less fatty acid breakdown and more lipid accumulation in the liver than controls.

Hepatic steatosis can also result from abnormal lipid transport. We were able to show that THS toxins result in a decrease in the expression and levels of apoB and MTTP, two key molecules involved in the assembly of VLDL particles. Apolipoproteins play a key role in transporting lipids from one organ to another. ApoB plays a key role in the transport of lipids out of the liver to peripheral tissue [18-26]. Decreasing the levels of this protein results in the decrease of transports of lipids out of the liver and consequently in the accumulation of lipids in the liver. Insulin has been associated with the negative regulation of apoB in the liver. Previously we have showed that THS-exposed mice have insulin resistance and high levels of insulin in the serum. Therefore is possible that
insulin resistance contributes to the low levels of apoB. The regulation of apoB also seems to occur at the transcriptional level since both DNA microarray and RNA-seq show that THS-exposed mice have lower expression levels of apoliproteins relative to the control. To our knowledge this is the first paper that shows that THS toxins causes lipid accumulation in the liver by targeting proteins involved in VLDL assembly (Figure 3.7A).

In conclusion, THS toxins decrease SIRT3 levels and IDH2 activity in the liver leading to a decrease in fatty acid metabolism (Figure 3.7B). In addition to a lower breakdown of fatty acids, THS-exposed mice also have lower VLDL levels compared to the control mice. These findings are significant because they provide further characterization of THS-induced hepatic steatosis in mice and further insights into the danger of exposure of THS toxins to liver health.
References


35. Lindholm CR, Ertel RL, Bauwens JD, Schmuck EG, Mulligan JD et al (2013). A high-fat diet decreases AMPK activity in multiple tissues in the absence of
hyperglycemia or systemic inflammation in rats. *Journal of physiology and biochemistry*, 1-11.


Figure 3.1. THS-exposure affects FA, TG and total lipid levels in the liver. Mice were exposed to THS toxins for six months before extracting the liver and serum for analysis (see Material and methods). THS-exposed mice have more (A) Free fatty acids in serum (B) TG in serum and (C) lipids in liver than control mice *= p< 0.05, ** p<0.01, ***p< 0.0006, NS = Not statistically significant. n=3 P values were adjusted for the number of times each test was run.
Figure 3.1
Figure 3.2. THS-exposure in conjunction Western Diet does not affects FA, TG and total lipid levels in the liver. THS-exposed mice placed on WD for five months before extracting the liver and serum for analysis (see Material and methods). THS-exposed mice placed on WD do not have a significant increase in (A) Free fatty acids, (B) TG levels, and (C) lipids compared to the THS-exposed mice on normal chow diet. * = p< 0.05, ** p<0.01, NS= Not statistically significant. n=3 P values were adjusted for the number of times each test was run.
Figure 3.2
Figure 3.3. THS-exposure in conjunction with AICAR treatment affects FA, TG and total lipid levels in the liver. THS-exposed mice treated with AICAR for eight weeks before the liver and serum was extracted for analysis. THS-exposed mice treated with AICAR have less (A) Free fatty acids (B) TG levels and (C) lipids than THS-exposed mice not treated with AICAR. *= p< 0.05, ** p<0.01, NS= Not statistically significant. n=3 P values were adjusted for the number of times each test was run.
Figure 3.3
Figure 3.4. Effects of THS-exposure on the levels of enzymes involved in fatty acid metabolism. Mice were exposed to THS toxins for six months before extracting the liver for analysis of enzyme levels (see Material and methods). (A-B) THS-exposed mice also have no significant change in acetyl-coA carboxylase (ACC) and carnitine palmitoyltransferase I (CPT1). (C) THS-exposed mice do not have lower long-chain acyl-CoA dehydrogenases (LCAD) levels or lower isocitrate dehydrogenase 2 (IDH2) levels than controls (D). * = p< 0.05, ** p<0.01, NS = Not statistically significant. n=3. P values were adjusted for the number of times each test was run.
Figure 3.4
Figure 3.5. THS toxins decrease IDH2 activity, SIRT3 and ATP levels in the THS-exposed mice. Mice were exposed to THS toxins for six months before extracting the liver for analysis of enzyme activity and levels (see Material and methods). (A) THS-exposed mice have lower IDH2 activity than control mice. (B) THS-exposed mice have lower SIRT3 levels and ATP levels (C) than control mice. *= p< 0.05, ** p<0.01 NS= Not statistically significant. n=10 for A and n=6 for B and C. P values were adjusted for the number of times each test was run.
Figure 3.5
Figure 3.6. THS exposure results in a decrease of VLDL assembly in the liver. Mice were exposed to THS toxins for six months before extracting the liver for analysis of enzyme levels (see Material and methods). (A) THS-exposed mice have lower levels of apoB in the liver than control mice. (B) THS toxins also result in lower MTTP protein in the liver of THS-exposed mice. (C) Lastly, THS-exposed mice also have lower levels of VLDL in the liver compared to the control mice. *= p< 0.05, ** p<0.01, ***p< 0.0006, NS= Not statistically significant. n=6 P values were adjusted for the number of times each test was run.
Figure 3.6
Figure 3.7. Plausible mechanism of how THS toxins alter VLDL assembly and fatty acid breakdown in the liver resulting in THS-induced hepatic steatosis. (A) THS decreases apoB resulting in TG accumulation in the liver (B) In addition to low levels of VLDL particles, THS-exposed mice also have lower fatty acid breakdown. THS-exposed mice have lower levels of SIRT3 and lower IDH2 activity and consequently lower fatty acid breakdown. Low levels VLDL and fatty acid breakdown lead to increase lipid accumulation in the liver and consequently development of THS-induced hepatic steatosis.
Figure 3.7
CHAPTER 4: Conclusion
The liver performs multiple metabolic functions and therefore compromising this organ can lead to the development of various diseases. Multiple non-genetic factors such as toxins, drugs, and diet can result in hepatic steatosis. To date, there is no treatment for hepatic steatosis and if is not detected and treated early it can progress to fibrosis, cirrhosis and hepatic carcinoma. A healthy lifestyle and cessation of exposure to toxins or drugs appears to be the key in reversing lipid accumulation in the liver and preventing hepatic steatosis. Evidence about the toxicity of THS toxins has been accumulating over the past years, however, not many studies have focused in the molecular and cellular damage of chronic exposure to this form of tobacco toxins. Moreover, virtually nothing is known about the molecular and cellular mechanism underlying the development of THS-induced hepatic steatosis.

I have provided a molecular and cellular mechanism of THS-induced hepatic steatosis in mice. I show that THS toxins lead to an increase oxidative stress in the liver resulting in THS-induce hepatic steatosis. THS-exposed mice have higher levels of oxidative-stress-mediated damage and more lipid accumulation in the liver. More importantly, when the THS-exposed mice were treated with antioxidants some of the cellular and molecular damage was reversed and the accumulation of lipids was decreased, suggesting that the damaging effects of THS toxins are mediated by a redox imbalance in these animals. Conversely, when THS-exposed mice were treated with APAP (Tylenol), oxidative stress increased as well as the accumulation of lipids.
I also show that exposure to THS toxins results in lower fatty acid metabolism and VLDL assembly. Treatment with AICAR, which is a common diabetes treatment, decreased fat accumulation in THS-exposed mice. THS-exposed mice have higher levels of fatty acids and TG and they were unable to process fatty acids by the TCA cycle. THS-exposed mice also have decreased VLDL assembly suggesting that the excess amount of lipids is not being transported out of the liver.

The findings presented here are novel and innovative because we (a) used a novel THS animal exposure system that mimics human exposure to THS in everyday life and (b) elucidated cellular and molecular mechanisms of lipid accumulation in the liver due to long-term THS exposure using mice as a model system. Virtually no exposure systems have been developed prior to the one proposed here for in vivo studies and the dangers of THS in the liver have also not been studied yet. As a consequence, these findings provide the basis and a model to study the adverse effects of THS toxins in the liver. They provide the basis for understanding the effects of THS toxins in the liver and they add to the accumulated evidence of the toxicity of THS. Additional studies need to be done to investigate whether, in addition to hepatic steatosis, exposure to toxins can lead to other diseases affecting the liver such as cancer and hepatitis.

Finally, the impact of the studies showed here is two fold: (a) They elucidate the mechanism by which THS-induced hepatic steatosis occurs so better treatments can be designed and (b) they will lead to studies in humans, which can then result in policymaking aimed at reducing exposure to THS toxins.