Cigarette Smoke Increases Cardiomyocyte Ceramide Accumulation and Inhibits Mitochondrial Respiration

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Cigarette Smoke Increases Cardiomyocyte Ceramide Accumulation and Inhibits Mitochondrial Respiration

Trevor Stanley Tippetts

A thesis submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Master of Science

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June 2015

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Cigarette smoking is a common and lethal worldwide habit, with considerable mortality stemming from its deleterious effects on heart function. While current theories posit altered blood lipids and fibrinogen metabolism as likely mediators, none have explored the role of the sphingolipid ceramide in exacerbating heart function with smoke exposure. Ceramide production is a consequence of cigarette smoke in the lung, and considering ceramide’s harmful effects on mitochondrial function, we sought to elucidate the role of ceramide in mediating smoke-induced altered heart mitochondrial respiration.

Lung cells were exposed to cigarette smoke extract and heart cells were exposed to the lung-cell conditioned medium. Adult male mice were exposed sidestream cigarette smoke for 8 weeks with dietary intervention and ceramide inhibition. Ceramides and heart cell or myocardial mitochondrial respiration were determined.

Lung cell cultures revealed a robust response to cigarette smoke extract in both production and secretion of ceramides. Heart cells incubated with lung-cell conditioned medium revealed a pronounced inhibition of myocardial mitochondrial respiration, though this effect was mitigated with ceramide inhibition via myriocin. In vivo, heart ceramides increased roughly 600% in adult mice with long-term sidestream cigarette smoke exposure. This resulted in a significant ceramide-dependent reduction in left myocardial mitochondrial respiration, as heart mitochondria from the mice exposed to both smoke and myriocin injections respired normally.

These results suggest ceramide to be an important mediator of altered myocardial mitochondrial function with cigarette smoke exposure. Thus, anti-ceramide therapies might be considered in the future to protect heart mitochondrial function with smoke exposure.

Keywords: ceramide, heart, cigarette smoke, mitochondria
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CHAPTER 1: Introduction

Cigarette smoke exposure is the leading cause of preventable deaths worldwide (WHO, 2011) and is currently one of the top ten contributors to the worldwide health burden (Deitel, 2003). Currently, there are a number of initiatives underway to reduce the prevalence of smoking. Despite the best effort of these initiatives, current trends indicate that the number of smokers will continue to increase worldwide (Ng et al., 2014; SAMHS, 2011). Cigarette smoke is also a commonly inhaled toxin. Almost half of the U.S. population is regularly exposed to cigarette smoke (Pirkle, Bernert, Caudill, Sosnoff, & Pechacek, 2006; Pirkle et al., 1996). Additionally, it is estimated that 20% of young children live with someone who smokes in their home (CDC, 2010). This alarming trend gives rise to the notion that cigarette smoking and related diseases will continue to be a world health burden. Exhaustive research is necessary to discover the full range of co-morbidities associated with cigarette smoking and a potential therapeutic method of treatment of related.

The Heart

Many of the health risks of smoking are associated with increased risks of chronic diseases. These diseases include cancer, emphysema, coronary heart disease, including cardiomyopathy (a deterioration of the heart muscle), and more (CDC, 2008; Lakier, 1992). Currently, cardiovascular disease is the leading cause of death in the United States (CDC, 2013). Cardiomyocytes are extremely metabolically active and highly oxidative cells. Because cardiomyocytes are highly oxidative, cardiomyocytes have a tremendous reliance on mitochondrial capacity (Lopaschuk & Jaswal, 2010). Altered function of mitochondria can directly lead to heart failure (Holmgren et al., 2003; Lesnefsky, Moghaddas, Tandler, Kerner, & Hoppel, 2001; Winter & Buist, 2000), which is a common consequence of cardiomyopathy.
As stated, the healthy function of mitochondria is essential to cardiomyocytes. Considering the importance of mitochondria in cardiomyocytes, pathologies and deleterious conditions that affect proper mitochondrial function in the heart are worth studying. Elucidating the factors that mediate altered cardiomyocyte mitochondrial physiology and the effects compounded by cigarette smoke exposure, a potent toxin, is a valuable area of study. Previous studies have observed that cigarette smoke inhibits mitochondrial respiratory function in lymphocytes (Miro et al., 1999) and myocardium (Knight-Lozano et al., 2002), but a mechanism for this inhibition has not been identified.

Ceramide

Ceramide is known as a sphingolipid. Sphingolipids were named after the enigmatic sphinx (Merrill et al., 1997). The functions of sphingolipids were unknown for a long period of time and thought to be a purely structural element of the cellular membrane (Simons & Ikonen, 1997). After extensive research, ceramide has been better characterized. Ceramide composed of sphingosine and a fatty acid and is synthesized from both a de novo pathway and a recycled pathway. Ceramide is synthesized de novo through an enzymatic cascade. Beginning with the substrates palmitoyl-CoA and serine, ceramide is produced through the reactions with the following enzymes: serine palmitoyltransferase (the rate-limiting enzyme in ceramide production), 3-ketosphingosine reductase, ceramide synthase, and dihydroceramide desaturase (Brice & Cowart, 2011).

In addition to the de novo pathway, ceramide is can be formed through a recycle pathway. Through the hydrolysis of sphingomyelin via sphingomyelinase enzymes, ceramide is formed (Claus, Dorer, Bunck, & Deigner, 2009). Some studies have explored the importance of the de novo pathway versus the recycle pathway. Gillard et al. showed that the importance of the
pathway depends on the cell type. Beyond the cell type, the current cell growth state and need for mitosis also regulates the reliance on each pathway. It was shown that as a cell grows, there is a greater need for de novo synthesis (Gillard, Clement, & Marcus, 1998).

As ceramide is studied further, distinct effects of ceramide have become evident. Ceramide accumulation in tissue was shown to induce apoptosis, programmed cell death (Haimovitz-Friedman, 1994). Additionally, an inhibition of ceramide production was shown to attenuate apoptosis (Dbaibo et al., 2001). As the known effects of ceramide continues to grow, all signs are pointing to the increased accumulation of ceramide leads to numerous pathologies. These pathologies associated with ceramide accumulation include obesity, metabolic syndrome, Alzheimer’s disease, and other pathologies (Yuyama, Mitsutake, & Igarashi, 2014).

The accumulation of ceramide may be an important factor in the development of cardiomyopathy. Ceramide has been shown to induce the release of proteins that induce apoptosis in the heart.

Cigarette Smoke

Cigarette smoke has been identified as a potent activator of inflammatory pathways in the lung (Reynolds et al., 2010). This inflammatory response has been shown to cause increased ceramide biosynthesis (Bikman, 2012; Holland et al., 2011). Ceramides are known to be important in the disruption of mitochondrial structure and function (Bikman & Summers, 2011; Gudz, Tserng, & Hoppel, 1997) which may possibly increase the risk of cardiomyopathy (Marin-Garcia, Goldenthal, Pierpont, & Ananthakrishnan, 1995). This leads to an intriguing model for potential ceramide accumulation in the heart. Because the heart is the next major anatomical structure after the lungs via the pulmonary vein, it is the downstream organ of pulmonary
ceramide release. The heart may receive a large and unhealthy dose of secreted ceramides from the lung in response to cigarette smoke exposure.

Diet

Additionally, obesity is a prevalent condition in the United States with approximately 35% of the adult population being obese (Ogden, Carroll, Kit, & Flegal, 2014). Diet and lifestyle are considered to be major factors in the prevalence of obesity (Wing et al., 2001). The western diet consists of high-sugar and high-fat content (Turnbaugh, Backhed, Fulton, & Gordon, 2008). The western diet has been linked to many pathologies. These pathologies include, but are not limited to; obesity, increased accrual of ceramide in tissues, gut microbiome changes, non-alcoholic fatty liver disease, atherosclerosis and others (Kasumov et al., 2015; Turnbaugh et al., 2008).

Whether or not cigarette smoking leads to reduced risks of obesity is a controversial subject. Conflicting studies have found that there may be evidence linking cigarette smoking to a greater risk of obesity (Chiolero, Faeh, Paccaud, & Cornuz, 2008; Thatcher et al., 2014) Obesity and an increase in sphingolipids leads to a greater risk for metabolic and cardiovascular disorders (Borodzicz, Czarzasta, Kuch, & Cudnoch-Jedrzejewska, 2015). With this in mind, a view of how cigarette smoke exposure and the western diet interact with the heart is worth studying.

Summary of Research

The research performed in our lab has generally focused on ceramide and its wide range of effects. Specifically, we have previously demonstrated that ceramides are secreted from A549 lung cells are potent producers of ceramide when exposed to cigarette smoke extract (Thatcher et al., 2014). Following the previously explained positioning of the lungs to the cardiovascular
system and by extension the rest of the body through the blood supply, we hypothesized that skeletal muscle would be affected by cigarette smoking either through the secreted ceramides or the dangerous metabolic cocktail of cigarette smoke. We were able to demonstrate that cigarette smoke exposure leads to an increased accumulation of ceramide in skeletal muscle (Thatcher et al., 2014).

This finding goes well with other previous discoveries in our lab regarding ceramide and mitochondrial function. The word mitochondria gives a clue as to how mitochondria are. Mitos means thread and chondria refers to a network. The mitochondria are a reticular network throughout the cell (Ishihara, Jofuku, Eura, & Mihara, 2003). A healthy mitochondria is a part of this network, or mitochondrion, while an unhealthy state of mitochondria involves fission that reduces the size of the reticular network. We found that ceramides stimulate mitochondrial fission via dynamin related protein 1 leading to reduced mitochondrial respiration and oxidative stress (Smith et al., 2013).

These discoveries, along with other research performed in our lab and by others, led us to investigate what happens to the heart in challenging conditions. The heart is a tissue highly reliant upon mitochondria (Lopaschuk & Jaswal, 2010) and perhaps the most important organ in the body. To the best of our knowledge, the study described in the following chapter is the first investigation into the interaction of the heart and cigarette smoke using ceramide as a potential mediator for disrupted mitochondrial respiration. In the study that follows, we aimed to identify the key by which cigarette smoke induces mitochondrial deficiencies in the heart.
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CHAPTER 2: Cigarette Smoke Increases Cardiomyocyte Ceramide Accumulation and Inhibits Mitochondrial Respiration

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Abstract

Background

Cigarette smoking is a common and lethal worldwide habit, with considerable mortality stemming from its deleterious effects on heart function. While current theories posit altered blood lipids and fibrinogen metabolism as likely mediators, none have explored the role of the sphingolipid ceramide in exacerbating heart function with smoke exposure. Ceramide production is a consequence of cigarette smoke in the lung, and considering ceramide’s harmful effects on mitochondrial function, we sought to elucidate the role of ceramide in mediating smoke-induced altered heart mitochondrial respiration.

Methods

Lung cells (A549) were exposed to cigarette smoke extract (CSE) and heart cells (H9C2) were exposed to the lung-cell conditioned medium. Adult male mice were exposed sidestream cigarette smoke for 8 weeks with dietary intervention and ceramide inhibition. Ceramides and heart cell or myocardial mitochondrial respiration were determined.

Results

Lung cell cultures revealed a robust response to cigarette smoke extract in both production and secretion of ceramides. Heart cells incubated with lung-cell conditioned medium revealed a pronounced inhibition of myocardial mitochondrial respiration, though this effect was mitigated with ceramide inhibition via myriocin. In vivo, heart ceramides increased roughly 600% in adult mice with long-term side stream cigarette smoke exposure. This resulted in a significant ceramide-dependent reduction in left myocardial mitochondrial respiration, as heart mitochondria from the mice exposed to both smoke and myriocin injections respired normally.
Conclusions

These results suggest ceramide to be an important mediator of altered myocardial mitochondrial function with cigarette smoke exposure. Thus, anti-ceramide therapies might be considered in the future to protect heart mitochondrial function with smoke exposure.

Background

Cigarette smoke exposure is the leading cause of preventable deaths worldwide (WHO, 2011) and is among the top ten contributors to the worldwide health burden (Deitel, 2003). Despite concerted social efforts to reduce smoking prevalence, current trends suggest the number of smokers will increase worldwide (Ng et al., 2014; SAMHS, 2011). Moreover, cigarette smoke is a common inhaled toxin—almost half of the U.S. population is regularly exposed to cigarette smoke (Pirkle, Bernert, Caudill, Sosnoff, & Pechacek, 2006; Pirkle et al., 1996) and approximately 20% of young children live with someone who smokes in the home (CDC, 2010). Much of smoking’s health burden stems from the increased risk of chronic diseases like cancer, emphysema, and cardiovascular disease (CDC, 2008; Lakier, 1992), including cardiomyopathy—a deterioration of heart muscle.

Cardiomyocytes are highly oxidative cells with a tremendous reliance on mitochondrial capacity (Lopaschuk & Jaswal, 2010), and altered mitochondrial function can lead to heart failure (Holmgren et al., 2003; Lesnefsky, Moghaddas, Tandler, Kerner, & Hoppel, 2001; Winter & Buist, 2000), a common consequence of cardiomyopathy. Considering the importance of healthy mitochondrial function in cardiomyocyte homeostasis, a valuable area of study is to elucidate the factors that mediate altered heart mitochondrial physiology and its effects with cigarette smoke exposure. Previous studies have observed that cigarette smoke exposure inhibits
mitochondrial respiratory function in blood cells (Miro et al., 1999) and myocardium (Knight-Lozano et al., 2002), but a mediating mechanism has yet to be identified.

Cigarette smoke has long been known to robustly activate inflammatory pathways in the lung (Reynolds et al., 2010), which increases ceramide biosynthesis (Bikman, 2012; Holland et al., 2011). Importantly, ceramides are known to disrupt mitochondrial structure and function (Bikman & Summers, 2011; Gudz, Tserng, & Hoppel, 1997), possibly increasing risk of cardiomyopathy (Marin-Garcia, Goldenthal, Pierpont, & Ananthakrishnan, 1995). Thus, the purpose of these studies was to determine whether the sphingolipid ceramide mediates cardiomyocyte mitochondrial disruption with cigarette smoke exposure. Considering the lung’s apposition with the environment, the lung is a logical site of external pathogen-induced stress, a product of which is ceramide biosynthesis (Holland et al., 2011). Moreover, given the heart’s location relative to pulmonary blood flow, the heart is a reasonable site of lung-derived ceramide uptake.

Methods

Cell Culture

Cigarette smoke extract (CSE) was generated as previously described with slight modifications (Reynolds, Cosio, & Hoidal, 2006). Briefly, one 2RF4 research cigarette (University of Kentucky, Lexington, KY) was continuously smoked by connecting the filtered end of the cigarette to a vacuum pump, pulling the particles into 5 ml of DMEM/F12 and the resulting medium was defined as 100% CSE. The total particulate matter content of 2RF4 cigarettes is 11.7 mg/cigarette, tar is 9.7 mg/cigarette, and nicotine is 0.85 mg/cigarette. Dilutions were made using DMEM/F12 + 10% FBS. Human type II–like pulmonary
adenocarcinoma cells (A-549; passage 10-15) were maintained in DMEM/F12 supplemented with 10\% FBS (Invitrogen) and antibiotics. Cells were split into 6-well dishes and grown to 80\% confluence. H9C2 cardiomyocytes were maintained in DMEM +10\% FBS. For differentiation into myotubes, cells were grown to confluency and the medium was replaced with DMEM +10\% horse serum (Invitrogen, Grand Island, NY). Myotubes were used for experiments on day 3 of differentiation. A-549 cultures were exposed to media supplemented with 10\% CSE or media alone for 4 h, after which the medium was transferred to differentiated H9C2 cardiomyocytes (termed “conditioned medium”) for 12 h. Where indicated, cells were treated with myriocin (10 \(\mu\)M, Sigma, M1177), a known and widely used inhibitor of ceramide biosynthesis. Muscle cells were harvested for RNA, protein, and lipid isolation following treatments. Cell survival during treatments was confirmed by trypan blue exclusion.

**Animals**

Male C57Bl6 mice were housed in a conventional animal house and maintained on a 12-hour light–dark cycle. Two animal studies were conducted. In the first study, animals received standard diet chow (Harlan Teklad 8604) and water *ad libitum*. At 12 wk of age, animals were randomly divided into room air and cigarette smoke (CS)-exposed groups. Mice were placed in soft restraints and connected to the exposure tower of a nose-only exposure system (InExpose System, Scireq, Canada). Animals were nasally exposed to sidestream CS generated by research cigarettes where a computer-controlled puff was generated every minute, leading to 10 min of CS exposure followed by 10 min of fresh air, repeated once. The CS-exposed group inhaled CS from two cigarettes per day for one week, at which point the dosage was increased to two cigarettes twice daily. Control animals were similarly handled and restrained in fresh air for the same duration. For the second study, 12-wk-old mice were separated into one of eight treatment
groups for eight weeks. Each of the following groups were duplicated to have one group receive
vehicle or myriocin injections (0.3 mg/kg) every other day for a total of eight groups: 1) control –
standard diet chow, no smoke; 2) cigarette smoke exposure (two cigarettes, twice daily) with
standard diet chow; 3) high-fat, high-sugar diet (Harlan Teklad 45F30S); 4) high-fat, high-sugar
diet with cigarette smoke exposure. Tissues were harvested at the conclusion of the study period.
Studies were conducted in accordance with the principles and procedures outlined in the
National Institutes of Health Guide for the Care and Use of Laboratory Animals and were
approved by the Institutional Animal Care and Use Committee (IACUC) at Brigham Young
University.

**Lipid Analysis**

For isolation of lipids, cell pellets or tissue were suspended in 900 μl ice-cold
chloroform/methanol (1:2) and incubated for 15 minutes on ice, then briefly vortexed. Separation
of aqueous and organic phases required addition of 400 μl of ice-cold water and 300 μl of ice-
cold chloroform. The organic phase was collected into a fresh vial, and lipids were dried in a
vacuum centrifuge (Eppendorf Concentrator Plus). Lipids were characterized and quantified
using a shotgun lipidomics technique on a Thermo Scientific LTQ Orbitrap XL mass
spectrometer. Evaporated lipid samples were re-suspended in a 2:1 chloroform: methanol Folch
solution (200 μL). The re-suspended lipids were then combined with a modified 2:1:1.25
chloroform: methanol: isopropanol Bligh and Dyer solution (800 μL) with 15 mM ammonium
acetate acting as an ionizing adduct. A 1.74 μM phosphatidylethanolamine, 1 μM C-17 ceramide,
1 μM tripalmitin internal standard cocktail (1 μL) was spiked into each sample for mass
calibration and characterization data alignment. Samples were analyzed using a 2.5-minute mass-
window scanning method in positive-ion mode at a resolution of 100,000 (FWHM at 400 m/z)
for all primary MS\textsuperscript{1} scans. MS\textsuperscript{2} fragmentation data was also collected (PQD at relative intensity of 35) and manually verified for each mass window to give additional confidence to the correct identification of abundant lipid species. Three technical replicate mass spectrometer runs were performed on each sample. Samples were injected at 8 \(\mu\text{L/min}\) using a direct-inject electrospray ionization (ESI) soft-ionization spray head from a Hamilton GASTIGHT glass syringe. A nitrogen sheath gas spray flow rate of 8 (arb. Units) was used for all runs. The spray voltage and capillary temperature were maintained at 5.0 KV and 275°C respectively. Each technical replicate was run in random order to reduce systematic bias. Data were analyzed using in-house developed peak summarization, recalibration, and lipid identification software using lipid database information from the LIPID Metabolites and Pathways Strategy (Lipid MAPS) Lipidomics Gateway database (Fahy et al., 2009). To ensure high-confidence identifications, an intensity threshold estimated to be 5% above instrumental static signal was implemented. Lipid identities were only assigned when significantly observable peaks were identified in at least two of the three technical replicate runs. Non-zero lipid quantities were averaged from the replicate runs. The lipid species identified across different ionization states or with adducts were totaled together. Quantification was completed by normalizing total ion counts to the relative abundance of the internal standard that was spiked into each sample.

Cell and Myocardium Permeabilization

For cells, H9C2 cardiomyocytes were detached in culture dishes with 0.05% trypsin-EDTA (Sigma) and growth medium was added to the culture. Contents were transferred to a tube and centrifuged for 10 min at 1000 \(\times\) g at RT. After removal of supernatant, cells were lifted in MiR05 [0.5 mM EGTA, 3 mM MgCl\textsubscript{2}, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 20 mM HEPES, 110 mM sucrose, and g/l BSA (Sigma; A3803) adjusted to pH 7.1] plus 1 mg/ml
digitonin and gently rocked at RT for 5 min before centrifugation at 1000 × g for 5 min. After discarding supernatant, cells were then suspended in 2.2 ml warm MiR05 and transferred to chambers in the O2K (Oroboros Instruments, Innsbruck, Austria). Following respiration protocol (outlined below), cells were removed from the chambers and used for further analysis, including protein quantification. For myocardial mitochondrial respiration, left ventricle was quickly removed from euthanized mice and immediately placed in ice-cold buffer X (60 mM K-MES, 35 mM KCl, 7.23 mM K2EGTA, 2.77 mM CaK2EGTA, 20 mM imidazole, 20 mM tuarine, 5.7 mM ATP, 15 mM PCr, 6.56 mM MgCl2-6H2O, pH 7.1) and trimmed of connective tissue. Small fiber bundles were prepared and gently separated along their longitudinal axis under a surgical scope (Olympus, ST) to 1-2 mg. Bundles were then transferred to a tube with chilled buffer X and 50 μg/ml saponin and rocked at 4°C for 30 min, then washed in buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH2PO4, 5 mM MgCl2-6H2O, 0.5 mg/ml BSA, pH 7.1) at 4°C for at least 15 min. Samples were then blotted dry and weighed.

*Mitochondrial Isolation*

To isolate mitochondria from left ventricle myocardium, the whole heart was placed in isolation buffer (300 mM sucrose, 10 mM Na-HEPES, 0.2 mM EDTA, pH 7.2) and the left ventricle isolated. After finely mincing the sample, trypsin (Sigma T9935) was added for two minutes before adding trypsin inhibitor (Sigma T9003). The sample was then transferred to a conical tube and allowed to settle. After removal of supernatant, samples were resuspended in isolation buffer with BSA (Sigma A3803) and homogenized with Teflon pestle before centrifugation (600 × g for 10 min at 4°C). Supernatant was then transferred to a new tube and centrifuged (8000 × g for 15 min at 4°C), then washed by gently adding isolation buffer and...
rotating the tube. A portion of this suspension is used for protein quantification. The pellet was then resuspended with isolation buffer + BSA.

**Mitochondrial Respiration Protocol**

High-resolution O$_2$ consumption was determined at 37°C in permeabilized cells and fiber bundles using the Oroboros O2K Oxygraph with MiR05 respiration buffer. Before addition of sample into respiration chambers, a baseline respiration rate was determined. After addition of sample, the chambers were hyperoxygenated to ~350 nmol/ml. Following this, respiration was determined by all or parts of the following substrate-uncoupler-inhibitor-titration (SUIT) protocol (Smith et al., 2013): electron flow through complex I was supported by glutamate + malate (10 and 2 mM, respectively) to determine basal oxygen consumption (GM$_B$). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GM$_P$). Succinate was added (GMS$_P$) for complex I + II electron flow into the Q-junction. To determine full electron transport system (F) capacity over oxidative phosphorylation, the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 μM, followed by 0.025 μM steps until maximal O$_2$ flux was reached). Complex II-supported ETS was then measured by inhibiting complex I with rotenone (Rot; 0.5 μM). Mitochondrial membrane integrity was tested in all experiments by adding cytochrome c (10 μM; GMc$P$). Lastly, residual oxygen consumption was measured by adding antimycin A (2.5 μM) to block complex III action, effectively stopping any electron flow, which provides a baseline rate of respiration. Where indicated, C2-ceramide (Sigma A7191; 20 μM) was added to respiration chambers.
Statistics

Data are presented as the mean ± SEM. Data were compared by ANOVA with Tukey’s post-hoc analysis (Graphpad Prism; La Jolla, CA). Significance was set at $p < 0.05$.

Results

Ceramide Inhibits Cardiomyocyte Mitochondrial Respiration

To determine the effects of ceramide on cardiac mitochondrial respiration, we utilized two models. First, mitochondria were isolated from left ventricle myocardium. During the course of the respiration protocol in the isolated mitochondria, C2-ceramide was added to one oxygraph chamber. The addition of ceramide (+C2) elicited a rapid and significant reduction in mitochondrial respiration that was sustained through maximal respiration with FCCP (Figure 2.1 A; see Methods for protocol details). A second model was the use of permeabilized intact left ventricle myocardium. One sample was incubated with C2-ceramide throughout the length of the protocol, which elicited a sustained reduction in mitochondrial respiration throughout the entire protocol (Figure 2.1 B).

Cigarette Smoke Extract Increases Lung Cell Ceramide Production and Secretion

The deleterious effect of cigarette smoking on heart function is undeniable, but the mechanism communicating the pulmonary insult to the heart is not clear. As proof of concept that the lung is capable of producing and secreting ceramides with smoke exposure, we treated A549 alveolar type 2 cells with cigarette smoke extract (CSE). Relative to PBS treatment, ceramides increased over 60% in CSE-treated lung cells, though this effect was mitigated with myriocin co-treatment (Figure 2.2 A). Further, CSE treatment elicited an almost three-fold increase in ceramide secretion into medium compared with PBS treatment (Figure 2.2 B). We
also observed an upward trend \( (P = 0.069) \) in circulating ceramides from whole blood following 5 d of sidestream smoke exposure in adult mice (Figure 2.2 C).

*Ceramide is Necessary for Smoke Extract-Induced Altered Cardiomyocyte Mitochondrial Disruption*

To determine the effects of lung cell-secreted ceramides on heart cell function, we utilized a conditioned medium *in vitro* model. Briefly, following incubation with PBS- or CSE-containing medium, with or without myriocin co-treatment, the conditioned medium from lung cells was transferred to H9C2 cardiomyotubes for 12 h. Following the incubation with conditioned medium, we determined cardiomyotube ceramide accrual and mitochondrial respiration. Ceramides were significantly increased in cardiomyotubes treated with conditioned medium from CSE-treated lung cells (Figure 2.3 A). However, when lung cells received treatment with myriocin in addition to CSE, the conditioned medium had no effect on heart cell ceramides (Figure 2.3 A). Mitochondrial respiration in the heart cells followed a similar trend as ceramides. Namely, respiration in heart cells receiving CSE conditioned medium was negatively affected, but not when myriocin was included in the lung cell culture medium (Figure 2.3 B).

*Ceramide Inhibition Prevents Reduced Myocardial Mitochondrial Respiration with Cigarette Smoke*

To more accurately determine the role of ceramides in mediating smoke-induced decayed heart mitochondrial respiration, we exposed animals to CS for 1 wk while receiving injections of PBS (vehicle) or myriocin every other day. Left ventricle ceramides increased four fold with CS compared with room air-exposed mice (Figure 2.4 A) with vehicle injections, though myriocin prevented this effect. Moreover, respiration was protected from CS in myriocin-injected animals (Figure 2.4 B).
Given the evidence suggesting the role of diet in increasing heart complications (Wilson, Tran, Salazar, Young, & Taegtmeyer, 2007) and ceramide accrual (Baranowski, Blachnio, Zabielski, & Gorski, 2007), we provided a high-fat, high-sugar (Western diet; WD) to animals in conjunction with smoke (or room air) exposure over the course of an 8-wk study. Similar to before, animals received vehicle (PBS) or myriocin injections every other day. In addition to observing a roughly 6-fold increase in heart ceramides with smoke exposure, we found an equally great increase in heart ceramides in animals receiving WD diet and smoke (Figure 2.5 A). However, ceramide inhibition was only partially successful in the WD + CS group. In general, ceramide accrual was associated with reduced myocardial mitochondrial respiration (Figure 2.5 B).

Discussion

Previous research demonstrated that smoke exposure contributes to cardiomyopathy (Gvozdjakova et al., 1984; Lough, 1978), which can be a consequence of altered mitochondrial function (Marin-Garcia et al., 1995), and we have recently shown that sidestream smoke alters mitochondrial function in skeletal muscle (Thatcher et al., 2014). Considering current worldwide smoking trends (Ng et al., 2014; SAMHS, 2011), cardiomyopathy and other cardiovascular burdens mediated by cigarette smoke are likely to increase. To date, the main instigators thought to mediate the heart-specific effects of smoking are altered blood lipids and changes in fibrinogen metabolism, (Cullen, Schulte, & Assmann, 1998; Newby et al., 1999), though the actual impact of these mechanisms is unknown (Cullen et al., 1998). While ceramides are known to mediate cellular disruption in the lung with smoking (Petrache et al., 2005), its impact on cardiomyocyte function with smoking has not been adequately explored. Thus, the purpose of this project was to determine the role of tobacco smoke-induced ceramides in disrupting
cardiomyocyte mitochondrial function. Our major discoveries were that lung cells secrete ceramide with sidestream smoke exposure and that ceramide accumulates in heart tissue and alters mitochondrial function.

To our knowledge, the first study to explore the effects of ceramide on mitochondrial respiration was published by the Hoppel laboratory, where they observed a rapid and robust inhibition of respiration in isolated heart mitochondria upon ceramide treatment (Gudz et al., 1997), and subsequent work corroborates these findings (Novgorodov & Gudz, 2009). We confirm those observations by Gudz et al. (Gudz et al., 1997) in isolated heart mitochondria and report similar findings in permeabilized left ventricle. Additionally, we have previously shown that ceramides substantially inhibit complex II action and that the general adverse effect of ceramides on mitochondrial respiration is dependent on ceramide-induced mitochondrial fission (Smith et al., 2013). Similarly, our findings of ceramide accrual in the lung with smoking corroborate those from other laboratories (Filosto et al., 2011; Levy, Khan, Careaga, & Goldkorn, 2009; Schweitzer et al., 2011), but while previous work focused on ceramide generation via sphingomyelinase, our effective use of myriocin suggests the importance of de novo ceramide synthesis in sidestream smoke-induced ceramide accumulation. However, while myriocin injections were sufficient to completely block ceramide accrual with smoking or diet separately, it was insufficient to prevent an increase in ceramides with combined smoking and diet. This may be a result of insufficient myriocin action in the midst of an overpowering stimulus (diet and smoke combined), or that sphingomyelinase may be particularly relevant in our combined treatment.

Importantly, we not only find increased ceramide production in lung cells with smoke exposure, but also ceramide release, providing proof of concept that the lung may be at least a
source of systemic ceramide accrual with smoke exposure. This is supported by our finding of an upward trend in circulating ceramides with smoke exposure. Nonetheless, ongoing experiments are testing the hypothesis that secreted ceramide by smoke-exposed pulmonary tissues travel and accumulate in cardiac muscle. Related to this, our conclusions that ceramide is the relevant component within the cultured medium upon transfer of medium from lung cells to cardiomyocytes is based on our use of myriocin. However, due to the harmful cocktail of molecules within the CSE, it is possible that a non-ceramide variable exists.

The level of ceramide accumulation we observe in the heart with smoking is substantial. We have previously quantified ceramide in various tissues (i.e., skeletal muscle, liver, brain) with dietary intervention (Bikman et al., 2012; Holland et al., 2011; Smith et al., 2013) and rarely observed greater than a roughly twofold increase in ceramides; similar changes have been observed in the heart (Baranowski et al., 2007). However, cigarette smoking appears to be a more robust inducer of systemic ceramide accumulation compared with diet. We found a roughly four-fold increase in heart ceramides after only 1 wk of smoke exposure (Figure 2.4) that increased to a six-fold change with an 8-wk exposure (Figure 2.5 A). Interestingly, supplementing the smoking regimen with a dietary component (Western diet, Harlan Teklad 45F30S) had no additive effect on heart ceramides (Figure 2.5 A).

Conclusions

In conjunction with our recent findings of altered skeletal muscle function with cigarette smoke exposure (Thatcher et al., 2014), the results of these studies implicate ceramide as an important mediator of myriad systemic metabolic effects. In particular, we find evidence that ceramides are a mediator of sidestream smoke-induced altered heart mitochondrial function.
While interventions to promote smoking cessation should continue, the increase in worldwide smoking and cardiovascular complications highlights the need for immediate therapies. Our findings suggest that ceramide inhibition may be a novel and potentially valuable therapeutic modality to protect heart function for those who are unwilling or unable to vacate smoke environments.
Figure 2.1: Ceramide Inhibits Left Ventricle Mitochondrial Respiration

A: Respiration rates of mitochondria isolated from left ventricle myocardium with addition of C2-ceramide during respiration protocol (20 μM; n = 4).

B: Mitochondrial respiration from permeabilized left ventricle myocardium (30 min) with continuous incubation with C2-ceramide (20 μM; n = 8). See Methods for more details on respiration protocol. *P <0.05.
Figure 2.2: Lung Cells Make and Secrete Ceramide in Response to Cigarette Smoke Extract

A: Ceramide levels in A549 cells treated with PBS- or 10% cigarette smoke extract-containing medium without (CSE) or CSE with myriocin (CSE + Myr), a ceramide inhibitor, for 12 h (n = 6).

B: Ceramides in culture medium of A549 cells following a 12-h treatment with PBS or CSE (n = 6).

C. Ceramides were determined from whole blood of adult mice following 5 d of room air (CON) or sidestream cigarette smoke (CS) ($p=0.069; n = 5$). *$p < 0.05$ for CSE vs. PBS.
Figure 2.3: Ceramide is Necessary for Decreased Mitochondrial Respiration in Myocardial Cells Following Treatment with Conditioned Medium from CSE-Treated Lung Cells

**A**: Ceramide levels in H9C2 cardiomyocytes treated with conditioned medium from A549 alveolar type 2 cells following incubation with normal growth medium (Con), Con with myriocin (Myr), cigarette smoke extract (CSE), and CSE with Myr (CSE + Myr) (n = 4).

**B**: Mitochondrial respiration from H9C2 cardiomyocytes following treatment in identical conditions (n = 5). See Methods for more details on respiration protocol. *P < 0.05 for CSE vs. Con.
Figure 2.4: Myriocin Prevents Left Ventricle Ceramide Accrual and Mitochondrial Dysfunction with Cigarette Smoke

Mice were exposed to room air (Con) or cigarette smoke (CS) for 1 wk while receiving PBS (vehicle) or myriocin injections every other day.

*A*: Ceramides were measured from left ventricle following treatment period (n = 6).

*B*: Mitochondrial respiration was reduced with CS treatment in vehicle-injected animals (n = 6). *P < 0.05 for CS vs all other treatments.
Figure 2.5: Western Diet Exerts a Minimal Effect on Heart Ceramides in Cigarette Smoke-Exposed Mice

Mice were exposed to room air (Con) or cigarette smoke (CS) for 8 wk while receiving PBS (vehicle) or myriocin injections every other day. Mice also received either a standard diet (SD) or Western diet (WD).

A: Ceramides were measured from left ventricle following treatment period (n = 4).

B: Mitochondrial respiration was determined from permeabilized left ventricle myocardium (n = 8). *P <0.05 for treatment vs. Vehicle SD. #P <0.05 for WD + CS myriocin vs. WD + CS vehicle.
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CHAPTER 3: General Discussion and Future Directions

The data from the previous chapter provides strong evidence that cigarette smoke is a potent ceramide synthesis agonist in cardiomyocytes. The increased ceramide accrual in the cardiomyocytes contributes to mitochondrial dysfunction via decreased mitochondrial respiration and potentially mitochondrial fission as shown previously by our lab (Smith et al., 2013). Mitochondrial function is essential for cardiomyocytes due to the highly oxidative nature of the heart. Our work builds on the previous work done by Knight-Lozano et al. showed that cigarette smoke leads to decreased mitochondrial respiration in myocardium (Knight-Lozano et al., 2002). This work suggests that ceramide plays the role of a mediating mechanism of cigarette smoke induced depression of cardiomyocyte mitochondrial respiration.

A salient point shown by the study involves ceramide and the left ventricular myocardium. Anatomically, the left ventricle is the powerful section of the heart with the responsibility to pump oxygenated blood to the rest of the body. Ceramide had been previously shown by Di Paola et al. to interact with the respiratory chain of mitochondria in the heart (Di Paola, Cocco, & Lorusso, 2000). We exposed isolated left ventricular myocardial mitochondria to ceramide because of the previously mentioned dynamic of cigarette smoke leading to the lungs and then directly from the lungs to the heart. We showed that ceramide added to isolated mitochondria leads to a depressed respiration in the maximum respiration state and the remaining states of respiration. Additionally, ceramide was shown to increase in mouse myocardium after 1 week of cigarette smoke exposure. We also performed a chronic treatment with cigarette smoke over an 8 week period. This extended treatment showed even greater ceramide accumulation in the left ventricular myocardium. Interestingly, when a western diet was introduced along with cigarette smoking, we saw a trend towards an additional accumulation of ceramide occurring.
The study presented in this thesis supports the model derived from previous work in our lab. This data has taken our past research and shown that cigarette smoke induces mitochondrial respiration deficiencies via ceramide. Additional work to fully elucidate the mechanisms and other factors involved with ceramide and mitochondrial respiration should be completed and will prove to be a novel area of research.

In line with our previous work, our data showed that ceramide was a necessary component for a significant reduction in left ventricular mitochondrial respiration. Using myriocin, a ceramide synthesis inhibitor, we were able to protect the heart against the effects of increased ceramides due to cigarette smoke exposure. Our success in protecting the tissue against ceramide accrual with myriocin suggests that de novo synthesis of ceramides is the key pathway used to produce ceramide in the study conditions. Further investigation into the importance of the various pathways of ceramide production may prove to be a key point of interest in finding therapeutic treatments for cigarette smoke induced cardiac mitochondrial complications.

The western diet slightly contributed towards increased levels of ceramide in the heart. Interestingly, we found that myriocin treatments were able to protect against ceramide accrual in the cigarette smoke and the western diet treatments when the two treatments were mutually exclusive. However, when the western diet and cigarette smoke treatments were combined, a significant accumulation of ceramides, compared to the myriocin control, cigarette smoke, and western diet groups occurred. This finding opens an area of further study. Our results from the mutually exclusive testing of cigarette smoke and western diet suggest that the de novo pathway in the synthesis of ceramide is the main contributor to ceramide accrual. Sphingomyelinase or other sources of ceramide (excluding the de novo pathway) may be important contributors in the combined insult of cigarette smoke exposure and the high fat-high sugar western diet.
Knowing that particulate insults such as cigarette smoke increase ceramide production in both the lung, heart, and other tissues, other avenues for research are appealing. With BYU residing in the Wasatch Front of Utah, an area consistently criticized for air pollution, exploring the effects of inversions and pollution on the heart would be a natural next step. Air pollution has long concerned the scientific community due to its effects on the heart, inflammation and other body systems (Brook et al., 2004). Air pollution has been shown to be associated with perturbations of heart rate and inflammatory responses (C. A. Pope, 3rd et al., 2006; C. A. Pope et al., 2004). Further investigation as to whether or not ceramide plays a role in these observed effects is warranted.

In conclusion, cigarette smoke up-regulates the production of ceramide in the heart. The enzymes that appear to be the major contributors to the extra synthesis of ceramide are the enzymes involved in the de novo synthesis pathway. The combined high fat-high sugar western diet and cigarette smoke treatment is able to overcome the myriocin inhibition of ceramide production in one of two ways. Either the ceramide accrual occurred through incomplete inhibition of the de novo pathway or a different pathway is contributing to ceramide production. The data presented in this thesis provides evidence that anti-ceramide treatments may be a unique, novel target for the prevention of cardiomyopathy or other pathologies of cardiac stress due to reduced mitochondrial function. At minimum, anti-ceramide treatments may be able to protect cardiac mitochondrial function where cigarette smoke exposure is occurring.
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Publications

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