The Role of Ceramides in Mediating Endotoxin-Induced Mitochondrial Disruption

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The Role of Ceramides in Mediating Endotoxin-Induced Mitochondrial Disruption

Melissa Ellen Smith Hansen

A dissertation submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The Role of Ceramides in Mediating Endotoxin-Induced Mitochondrial Disruption

Melissa Ellen Smith Hansen
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Ceramides are sphingolipids that serve as important second messengers in an increasing number of stress-induced pathways. Ceramide has long been known to affect the mitochondria, altering both morphology and physiology. Lipopolysaccharide (LPS) is a prevalent circulating inflammatory agent in obesity, potentially mediating some of the pathologies associated with weight gain. Given previous findings of TLR4-mediated ceramide accrual and ceramide-mediated mitochondrial disruption, we questioned whether ceramide is necessary for LPS-induced mitochondrial disruption. We found that LPS treatment increased gene transcript levels of ceramide synthesis enzymes and mitochondrial fission proteins and increased ceramide content in cultured myotubes and in mouse tissue. Mitochondrial respiration from permeabilized red gastrocnemius was reduced from animals receiving LPS injections when compared with those receiving vehicle (PBS). However, respiration from mice receiving both LPS and myriocin, a ceramide inhibitor, (0.3 mg/kg) was similar to PBS-injected animals. We treated murine myotubes with similar LPS conditions. These cells demonstrated increased ceramide synthesis and increased levels of mitochondrial fission with LPS treatment; these effects were mitigated with the addition of myriocin. However, in contrast to the whole gastrocnemius response in animals receiving LPS, respiration from myotubes was increased with LPS alone, and even higher with both myriocin alone and myriocin with LPS.

We also sought to assess the impact of ceramide on skeletal muscle mitochondrial structure and function. A primary observation was the rapid and dramatic division of mitochondria in ceramide-treated cells. This effect is likely a result of increased Drp1 action, as ceramide increased Drp1 expression and Drp1 inhibition prevented ceramide-induced mitochondrial fission. Further, we found that ceramide treatment reduced mitochondrial O2 consumption (i.e., respiration) in cultured myotubes and permeabilized red gastrocnemius muscle fiber bundles. Ceramide treatment also increased H2O2 levels and reduced Akt/PKB phosphorylation in myotubes. However, inhibition of mitochondrial fission via Drp1 knockdown completely protected the myotubes and fiber bundles from ceramide-induced metabolic disruption, including maintained mitochondrial respiration, reduced H2O2 levels, and unaffected insulin signaling. These data suggest that the forced and sustained mitochondrial fission that results from ceramide accrual may alter metabolic function in skeletal muscle, which is a prominent site not only of energy demand (via the mitochondria), but also of ceramide accrual with weight gain.

Keywords: ceramide, LPS, endotoxemia, mitochondria, mitochondrial fission, metabolic disruption
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CHAPTER 1: Review of the Literature

One of the most disturbing epidemics in the United States is the unabated rise in obesity. According to 2010 data from the Centers for Disease Control, over 78 million American adults are obese—more than one third of the US adult population (Ogden 2012). If current trends continue, over half of the US population will be obese by 2030 (Velkoff 2000). In 1998, healthcare costs related to obesity were estimated to be $78.5 billion. This number rose to $148 billion by 2008, a nearly twofold increase in only ten years (Finkelstein, Trogdon et al. 2009). Dietary excess, poor nutrient quality, and reduced physical activity combine to increase the risk of developing insulin resistance—the foundation of type 2 diabetes, as well as a contributing cause and consequence of obesity (Hotamisligil, Peraldi et al. 1996, Kimokoti, Gona et al. 2012)—as well as other metabolic issues. The current national and worldwide increases in the prevalence of obesity, type 2 diabetes, and metabolic syndrome give rise to the notion that this is a complex problem and intensive research is necessary to discover effective therapeutic interventions. There is an urgent need for innovative solutions to curb this upward trend.

Many distinct explanations have been proposed regarding the etiology of obesity-induced metabolic disruption, defined here as decreased mitochondrial oxygen consumption, increased mitochondrial reactive oxygen species (ROS) production, and decreased insulin sensitivity. Three prevailing ideas include: 1) the inflammation hypothesis, which focuses on increased circulating levels of both exogenous and endogenous proinflammatory substances that activate signaling pathways that lead to metabolic disruption; 2) the lipotoxicity hypothesis, which posits that toxic, atopic accumulation of intracellular lipids leads to metabolic disruption; and 3) the mitochondrial fission hypothesis, which presumes fractionated mitochondria lead to metabolic deterioration and disruption (Taubes 2009, Jheng, Tsai et al. 2012, Lee, Jee et al. 2013).
Inflammation

Obesity has been well characterized as a low-grade, subclinical inflammatory state (Greenberg and Obin 2006, Lumeng and Saltiel 2011, Bradlow 2014). Proinflammatory substances come from many sources in obesity. Some are endogenous cytokines. These can be produced by macrophages that have invaded fat tissue, particularly in the viscera, and become engorged and overloaded by nutrients, subsequently becoming proinflammatory, and releasing chemotactic cytokines (referred to as adipokines). The release of adipokines recruits additional macrophages to infiltrate the area, leading to low-grade chronic inflammation (Lee, Jee et al. 2013). As a result, the inflamed adipose tissue secretes additional inflammatory cytokines into the blood stream, including tumor necrosis factor-α (TNF-α) (Hotamisligil, Shargill et al. 1993), interleukin-6 (IL-6) (Bastard, Jardel et al. 2000, Fernandez-Real, Vayreda et al. 2001) and monocyte chemoattractant protein-1 (MCP-1) (Takahashi, Mizuarai et al. 2003, Xu, Barnes et al. 2003). These cytokines have been shown to promote insulin resistance, disrupt cellular respiration, and increase ceramide de novo synthesis in multiple tissues, namely the adipose, muscle, liver and pancreatic beta cells (Higuchi, Proske et al. 1998, Kern, Ranganathan et al. 2001, Klover, Zimmers et al. 2003, Rotter, Nagaev et al. 2003, Kamei, Tobe et al. 2006, Sell, Dietze-Schroeder et al. 2006, Maury and Brichard 2010, Holland, Bikman et al. 2011).

In addition to the endogenous cytokines released as a consequence of obesity, exogenous inflammatory substances are also elevated in the blood of obese individuals. The most notable of these is lipopolysaccharide (LPS) derived from the cell walls of gram negative bacteria. Many of these bacteria make up the microbiota that populates the gut. In adult humans, the microbiota is dominated by the bacterial phyla Firmicutes, which are primarily gram positive, and Bacteroidetes, which are primarily gram negative. These bacteria aid in the digestion of many
plant saccharides into short chain fatty acids, which are more easily absorbed by the mammalian small intestine. (Ley, Turnbaugh et al. 2006, Turnbaugh, Ley et al. 2006, Kelly, Colgan et al. 2012) The regular turnover of these bacteria, as well as additional ingested bacteria, results in a stable supply of LPS in the small intestine (Kelly, Colgan et al. 2012). Several groups have demonstrated that high-energy meals increase blood levels of LPS (Ghanim, Abuaysheh et al. 2009, Deopurkar, Ghanim et al. 2010, Laugerette, Vors et al. 2011). This phenomenon is known as post-prandial endotoxemia. High fat diets in particular have been shown to cause this effect, up to a 50% increase in serum LPS (Erridge, Attina et al. 2007). This increase is thought to result from a combination of increased gut permeability, and increased transcellular transport of LPS through gut epithelial cells. In a 2008 study, Cani et al. demonstrated increased gut permeability to an inert fluorescent molecule in high-fat fed mice that was not observed in controls (Cani, Bibiloni et al. 2008). These animals had decreased expression of tight junction proteins. Additional studies found transient epithelial injury as a result of emulsified fat ingestion (Kvietyts, Specian et al. 1991). There is also evidence that LPS is transported from the lumen of the small intestine into the blood via chylomicron transport. Inhibition of chylomicron formation blocks the postprandial increase in LPS (Ghoshal, Witta et al. 2009).

The increase in blood levels of LPS becomes relevant in a scenario that has been termed ‘metabolic endotoxemia.’ Elevated blood LPS stimulates many pathologies with the combined outcome of metabolic syndrome. Low-dose, sub-septic LPS treatment of mice over a period of four weeks has been shown to cause obesity and insulin resistance to a similar extent as in high-fat-fed mice over the same period of time (Cani, Amar et al. 2007). A human study conducted in Finland found that elevated blood levels of LPS were significantly associated with obesity, metabolic syndrome, diabetes, and coronary heart disease. (Kallio, Hatonen et al. 2014)
These metabolic effects of LPS can be, at least in part, explained by activation of Toll-like Receptor 4 (TLR4) activation. When LPS is in circulation, it binds to LPS binding protein (LBP) and together they can then bind CD14. Once the LPS/LBP/CD14 complex has been established, it can bind to and activate TLR4. TLR4 is a membrane receptor, and its activation has many downstream targets and effects. Frisard et al. found that metabolic endotoxemia activation of TLR4 resulted in decreased fatty acid oxidation as measured by $^{14}$CO$_2$ production from the oxidation of [1-$^{14}$C]palmitic acid (Frisard, McMillan et al. 2010). TLR4 activation has also been found to increases de novo synthesis of ceramide (Holland, Bikman et al. 2011) as well as many endogenous proinflammatory cytokines such as TNFα and IL-6. Mitochondrial ROS production increases when TLR4 is activated in vitro (Yuan, Zhou et al. 2013).

**Lipotoxicity**

In addition to altered inflammation, the second factor that may explain obesity-induced metabolic disruption is lipotoxicity. Obesity predisposes individuals to metabolic diseases because the oversupply of lipids to tissues not suited for fat storage leads to lipid accumulation known as lipotoxicity. This leads to the accumulation of fat-derived molecules that impair tissue function (Siddique, Li et al. 2013).

**Ceramide**

One such fat-derived molecule is the sphingolipid ceramide. Ceramide is known to accumulate in insulin-sensitive (Holland, Bikman et al. 2011) as well as high-metabolic-rate organs (Giusto, Roque et al. 1992, Lightle, Oakley et al. 2000, Holland, Bikman et al. 2011, Bikman, Guan et al. 2012), eliciting a potentially powerful impact on whole-body metabolic function. Ceramide is known to induce insulin resistance via decayed Akt/PKB phosphorylation, as well as increase the generation of mitochondria-derived reactive oxygen species (ROS) (Kim,
Intracellular ceramide is derived from three sources: hydrolysis of sphingomyelin, a phospholipid in cell membranes; \textit{de novo} synthesis; and the breakdown of other complex sphingolipids, known as the salvage pathway.

De novo synthesis begins when a palmitate molecule and a serine molecule are joined in a condensation reaction catalyzed by the enzyme serine palmitoyl transferase (SPT) to form 3-keto-sphinganine. This is the rate-limiting step of ceramide synthesis.

In 1972 Kleupfel et al. discovered an antifungal molecule derived from the mycelium of the thermophilic fungus \textit{Myriococcum albomyces}. They called the compound ‘myriocin’. Their findings include the observation that “The compound appears to be too toxic for therapeutic purposes.” (Kleupfel 1972) It wasn’t until 1995 that researchers in Japan noticed the structural similarities between myriocin and sphingosine and proposed that this compound could be useful in studying the sphingolipid pathway (Mikaye 1995). Myriocin is a potent inhibitor of SPT and thus a blocker of \textit{de novo} ceramide synthesis.

\textit{Mitochondrial Fission}

Despite being commonly represented as small, segmented structures, mitochondria are highly dynamic organelles undergoing continuous fission and fusion. The word \textit{mitochondrion} derives from two Greek words: \textit{mitos} for ‘threads’ and \textit{chondron} for ‘grains’. Fission results in the production of short mitochondrial rods or spheres and fusion promotes the formation of long, filamentous mitochondria (Zhan, Brooks et al. 2013). The normal roles of fission in a cell include transmitting mitochondria among dividing cells and meeting increased ATP demands. Fission also plays a key role in maintaining mitochondrial quality as it allows dysfunctional
mitochondria to be severed from the network and to be removed by autophagy (Peterson, Johannsen et al. 2012). Typically there is a balance of fission and fusion in mammalian cells with both processes occurring at similar rates (Yoon, Galloway et al. 2011). Because mitochondria have two membranes, fission and fusion are both two-step processes that are regulated by proteins on both the inner and outer mitochondrial membranes. For example, during fusion optic atrophy gene 1 (OPA1) is located on the inner membrane and mitofusin 2 (Mfn2) is located on the outer membrane. Disrupting either one of these proteins is sufficient to prevent mitochondrial fusion. (Zorzano, Liesa et al. 2009, Zhao, Lendahl et al. 2013)

An increasing number of studies suggest that mitochondrial structure as determined by fission and fusion determines mitochondrial function, and an imbalance of fission and fusion events impacts a broad range of cellular processes (Liesa, Palacin et al. 2009, Yoon, Galloway et al. 2011, Zhao, Lendahl et al. 2013). Interventions to stimulate either mitochondrial fission or fusion elicit disparate effects on metabolic function, such as mitochondrial respiration (Westermann 2012) and insulin signaling (Jheng, Tsai et al. 2012). In a 2005 study, obese individuals and individuals with T2DM had reduced skeletal muscle fusion protein expression compared to control subjects (Bach, Pich et al. 2003). A consensus suggests mitochondrial fusion as beneficial in maintaining insulin sensitivity and resisting fat gain with dietary challenge (Jheng, Tsai et al. 2012, Sebastian, Hernandez-Alvarez et al. 2012).

Reactive Oxygen Species

In the electron transport system (ETS) within the mitochondria, electrons are shuttled from complex to complex in a series of redox reactions that provide the potential energy necessary to create a proton gradient in the intermembrane space. This proton gradient is utilized for ATP synthesis. At the end of the ETS, oxygen is the final electron acceptor. This can be a
costly process as it results in the formation of oxygen radicals, which cause subsequent oxidative
damage. Several ROS species are present intracellularly: the superoxide radical, \( \text{O}_2^- \); hydrogen
peroxide, \( \text{H}_2\text{O}_2 \); and the hydroxyl radical, \( \text{OH}^- \). The mitochondria have many mechanisms in
place to manage radical concentrations, including enzymes such as superoxide dismutase (SOD),
catalase, glutathione peroxidase, and peroxiredoxin III (Fridovich 1997), however when ROS
concentrations overcome these mechanisms it leads to mitochondrial swelling and release of
cytochrome c, leading to apoptosis. Increased intracellular ROS concentrations may be an
indication of an increase in mitochondrial uncoupling, exogenous stressors, or high work
conditions (Aon, Cortassa et al. 2010). Increase in intracellular ROS has been shown in many
pathological states, including aging and neurodegenerative diseases, to lead to mitochondrial

Proposed Model

With these three distinct theories in mind (i.e., inflammation, lipotoxicity and
mitochondrial fission), we sought to test the unifying hypothesis that LPS mediated metabolic
endotoxemia induces ceramide synthesis, and the resulting intracellular accumulation of
ceramide triggers mitochondrial fission, which is a critical mediator of ceramide-induced
metabolic dysfunction.

Skeletal Muscle—An Ideal Metabolic Model

In humans, skeletal muscle is the largest organ by weight, making up more than 50% of
the total body mass in an adult (Zorzano, Liesa et al. 2009). In addition to its obvious functions
of locomotion and posture, its highly dynamic metabolic rate and tissue mass gives it an
important role in heat production (when warranted) and management of whole body metabolism
(Zorzano, Liesa et al. 2009, Wang, Winters et al. 2012). For this reason, these studies focus on
skeletal muscle, including muscle cell cultures and rodent skeletal muscle tissue.

Objectives

The purpose of this dissertation was to identify the role of ceramide in mitochondrial dynamics and to establish whether or not ceramide is a mediator in the effects of inflammation on mitochondrial function.

The specific purpose of Chapter 2 was to determine the role of ceramide as a mediator of LPS induced altered mitochondrial function in both murine myotubes and in an in vivo mouse model of chronic sub-clinical inflammation. We hypothesized:

1) Ceramide is necessary for inflammation-induced altered mitochondrial respiration
2) The inhibition of de novo ceramide synthesis would reduce or eliminate the deleterious effect of LPS on mitochondrial respiration and mitochondrial dynamics

Experiments in Chapter 2 determined the effect of LPS treatment on the fission/fusion state of skeletal muscle mitochondria and on metabolic function.

The specific purpose of Chapter 3 was to determine whether mitochondrial fission is necessary for ceramide-induced mitochondrial defects in both cell culture experiments and high fat fed mice. We hypothesized:

1) Ceramide accumulation in skeletal muscle modulates the composition and actions of proteins involved in mitochondrial form and structure
2) Accumulation of ceramide in skeletal muscle would reduce energy utilization by the muscle and the whole-body by regulating mitochondrial function.

We sought to determine the impact of both ceramide accumulation and ceramide depletion on metabolic rate. Experiments in Chapter 3 helped provide understanding as to
ceramide’s effect on muscle and whole-body metabolic function.
CHAPTER 2: Ceramide is Necessary for \textit{In vivo} LPS-Induced Mitochondrial Disruption

\textit{Abstract}

Lipopolysaccharide (LPS) is a prevalent circulating inflammatory agent in obesity, potentially mediating some of the pathologies associated with weight gain. Given previous findings of TLR4-mediated ceramide accrual and ceramide-mediated mitochondrial disruption, we questioned whether ceramide is necessary for LPS-induced mitochondrial disruption. We found that tissue from mice receiving daily intraperitoneal LPS injections (0.1 mg/kg) for four wk had increased gene transcript levels of ceramide synthesis enzymes and mitochondrial fission proteins, and increased ceramide content. Mitochondrial respiration from permeabilized red gastrocnemius was reduced from animals receiving LPS injections when compared with those receiving vehicle (PBS). However, respiration from mice receiving both LPS and myriocin, a ceramide inhibitor, (0.3 mg/kg) was similar to PBS-injected animals. In order to determine the direct effect of LPS on cultured myotubes, we treated murine myotubes with similar conditions as animal injections. These cells demonstrated increased ceramide synthesis and increased levels of mitochondrial fission with LPS treatment; these effects were mitigated with the addition of myriocin. However, in contrast to the whole gastrocnemius response in animals receiving LPS, respiration from myotubes was increased with LPS alone, and even higher with both myriocin alone and myriocin with LPS.

\textit{Introduction}

In the past 100 years, the prevalence of obesity has been increasing in every age, race, sex and socioeconomic group (McAllister, Dhurandhar et al. 2009). The co-morbidities associated with obesity include insulin resistance, type 2 diabetes, gall bladder disease, osteoarthritis, sleep apnea, gout, dyslipidemia, steatosis, hypertension, heart disease, stroke and
cancer (Khaodhiair, McCowen et al. 1999, Pi-Sunyer 2009). Many mechanisms have been proposed to explain the link between obesity and the many related pathologies. One compelling and well-corroborated explanation is the proinflammatory state that is both cause and consequence of increased weight gain. Obesity is associated with increased levels of myriad proinflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and others (Hotamisligil, Shargill et al. 1993, Bastard, Jardel et al. 2000, Fernandez-Real, Vayreda et al. 2001). These individuals also exhibit elevated blood levels of exogenous proinflammatory endotoxins, such as LPS.

The increased blood levels of lipopolysaccharide (LPS) in individuals following high energy meals (Ghanim, Abuaysheh et al. 2009, Deopurkar, Ghanim et al. 2010, Laugerette, Vors et al. 2011), as well as the chronic elevation of blood LPS in obese persons leads to activation of toll-like receptor 4 (TLR4). TLR4 activation has been previously shown to increase de novo ceramide synthesis (Holland, Bikman et al. 2011). Ceramides are well-established disrupters of metabolic function, being linked to impaired insulin signaling and apoptosis. In the present study, we examined the hypothesis that ceramides are an essential mediator of LPS-induced metabolic disruption, particularly altered mitochondrial structure and function.

Methods

Animals

All experiments were performed on male C57Bl/6 mice between 8 and 20 weeks old. 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. All animals were housed in a temperature controlled (20-21°C) environment with a
12hr:12hr light-dark cycle. Water and food were administered *ad libitum*.

**Myriocin Administration**

Myriocin supplementation was delivered via intraperitoneal injections three times per week at a dosage of 0.3 mg/kg.

**Lipopolysaccharide Administration**

Mice in LPS treatment groups received daily intraperitoneal injections of LPS at a dosage of 0.1 mg/kg for a period of 28 d.

**C2C12 Cells**

C2C12 murine myoblast cells were maintained in DMEM plus 10% fetal bovine serum (Invitrogen). For differentiation into myotubes, C2C12 myoblasts were grown to confluency and the medium was replaced with DMEM plus 10% horse serum (Invitrogen). Myotubes were used for experiments on day 4 of differentiation.

**Cell Treatments**

For LPS treatment, O55:B5 lipopolysaccharide from *e. coli* (Sigma-Aldrich; L2880) was diluted in ddH₂O in a 1mg/ml stock. For myriocin treatment, myriocin (Sigma-Aldrich; M1177) was diluted in methanol in a 5mM stock solution.

**Lipid Analysis**

For ceramide specific lipid isolation, homogenized tissues were re-suspended in 300 μl ice-cold PBS and 1.5 mL of methanol. 500 pmol of internal C-17 ceramide (Avanti Lipids; 860647) standard was spiked into each sample. Samples were centrifuged and supernatant was transferred to a clean tube. Following the addition of 30 μl of 1 M KOH in methanol, samples
were incubated overnight at 50°C. Samples were dried to 50% volume and 25 μl glacial acetic acid was added to neutralize KOH. Separation of aqueous and organic phases required addition of 300 μl LC-grade chloroform and 600 μl DDH2O followed by centrifugation for 2 minutes at maximum speed. The lower organic phase was transferred to a fresh vial. This separation step was repeated twice. All lipid samples 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 internal standard (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 60,000 (fwhm at 400 mz) for all primary MS1 scans. MS2 fragmentation data was also collected 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 10 μL/min using a direct-inject electrospray ionization (ESI) soft-ionization spray head from a Hamilton GASTIGHT glass syringe. The spray voltage and capillary temperature were maintained at 5.0 KV and 275oC 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
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.

Confocal Microscopy

C2C12 myoblasts were grown to confluence in chamber slides (NUNC Lab-Tek II Chambered Coverglass System; 155382) and differentiated at day 4. The mitochondrial dye MitoTracker Red CMXRos (Molecular Probes; M7512), dissolved in anhydrous dimethylsulfoxide (DMSO), was added to cultured myotubes at a concentration of 250 nM. The cells were incubated for 30 minutes at 37°C in the dark and then visualized using a confocal microscope (Olympus IX81).

Quantitative Real-time PCR

Total RNA was extracted and purified from tissues using TRIzol (Invitrogen) according to the manufacturer’s recommendations. cDNA was synthesized from mRNA via reverse-transcriptase PCR using a commercial cDNA synthesis kit with oligo(dT) primers (iScript Select cDNA Synthesis; Bio-Rad). Quantitative real-time PCR was performed with Evagreen Ssofast (Bio-Rad) using a BioRad CFX Connect Real-Time PCR Detection system. Primer sequences were 5’- ACAGGATGCAGAAGGAGATTAC and 5’- CACAGAGTACTTGCGCTCAGGA as forward and reverse primers for actin, 5’-ACTTGACCTCCCTACTGGC and 5’-TCCTCTATCCCCGTTGACACC as forward and reverse primers for Drp-1, and 5’-
AAGTCCGGGAAAGCTGAAAGT, 5’-TCTCGGTTATGGAACCAACC as forward and reverse primers for Mfn2. β-Actin reactions were performed side by side with every sample analyzed. Changes in mRNA level of each gene for each treatment were normalized to that of the β-actin control mRNA according to Pfaffl (Pfaffl 2001).

Cell and Muscle Fiber Bundle Permeabilization

For cells, C2C12 myotubes 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 rpm at RT. After removal of supernatant, cells were resuspended in mitochondrial respiration buffer 05 (MiR05; 0.5 mM EGTA, 10 mM KH2PO4, 3 mM MgCl2-6H2O, 60 mM K-lactobionate, 20 mM HEPES, 110 mM Sucrose, 1 mg/ml fatty acid free BSA, pH 7.1) plus 1 mg/ml digitonin and gently rocked at RT for 5 min before centrifugation at 1000 rpm 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). Following respiration protocol (outlined below), cells were removed from the chambers and used for further analysis, including protein quantification. For skeletal muscle use, red gastrocnemius was quickly removed from mice following cervical dislocation 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 taurine, 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 a size of around 1 mg. Bundles were then transferred 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.
Mitochondrial Respiration

High-resolution O$_2$ consumption was determined at 37°C in permeabilized cells (MiR05) and fiber bundles (buffer Z) using the Oroboros O2K Oxygraph. 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 according to two substrate-uncoupler-inhibitor-titration (SUIT) protocols (Jheng, Tsai et al. 2012). For SUIT1, to determine general electron transport system function, electron flow through complex I was supported by glutamate+malate (GM; 10 and 2 mM, respectively) to determine oxygen consumption from proton leak (GML). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GMP). The integrity of the outer mitochondrial membrane was then tested by adding cytochrome c (10µM; GMcP). Succinate was added (GMSP) for complex I+II electron flow into the Q-junction. To determine full electron transport system (ETS) 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 O2 flux was reached). Complex II-supported ETS was then measured by inhibiting complex I with rotenone (Rot; 0.5 µM). Lastly, residual oxygen consumption was measured by adding antimycin A (2.5 µM) to block complex III action, effectively stopping any electron flow. This provides a rate of respiration that is used as a baseline and all presented values are controlled for by using this baseline. SUIT2 was used to determine complex II-specific respiration. This SUIT involved succinate (10 mM) with the addition of rotenone (0.5 µM) for complex II-mediate leak respiration (S(Rot)L) and then ADP (2.5 mM) for complex II-mediated oxidative phosphorylation. Lastly, maximal ETS was determined by FCCP addition as before. Similarly, residual oxygen consumption was determined
by antimycin A (2.5 µM) addition and all values were controlled for by this value.

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

LPS Increases Muscle Ceramides

Mice who received daily LPS injections for a period of four weeks experienced a nearly two-fold increase in ceramide levels in soleus muscle However, ceramide inhibition via myriocin injection (an SPT inhibitor) mitigated this effect (fig. 2.1). When assessing the effects of LPS injections on gene expression, LPS increased the mRNA expression of serine palmitoyltransferase (SPT) 1 and 2 (fig. 2.2)

In an in vitro model of metabolic endotoxemia, murine myotubes were treated with a low dose of LPS. LPS treatment resulted in a slight, but significant increase in ceramides, but not when co-treated with myriocin (fig. 2.3). The increased ceramides may be a function of enhanced SPT; SPT1 transcript was significantly increased with LPS treatment (fig. 2.4).

LPS Induces Mitochondrial Fission

Mitochondrial structure was determined via confocal microscopy in murine myotubes following LPS treatment. 16 h of LPS incubation was sufficient to cause obvious fission. Once again, these effects appeared to be ceramide dependent as addition of myriocin was sufficient to prevent LPS-induced mitochondrial fission (fig. 2.5B).

In mice that received LPS injections for 28 d, transcript levels of dynamin-related protein 1 (Drp1) were increased; Drp1 is a mitochondrial protein that mediates fission. Co-treatment
with LPS and myriocin had a protective effect on Drp1 transcription (fig. 2.5A). These results indicate that LPS-induced mitochondrial fission is ceramide mediated and may involve Drp1 signaling.

**LPS Alters Mitochondrial Respiration**

Mitochondrial respiration was measured from permeabilized red gastrocnemius following the study protocol. Muscle tissue from the LPS-treated mice exhibited reduced mitochondrial respiration when compared with vehicle-injected mice (fig. 2.6). Myriocin injections alone had no effect compared to vehicle, but mice that received myriocin injections in conjunction with LPS injections had improved respiration compared to LPS injections alone, suggesting an important role for ceramide in mediating LPS-induced reduced respiration in whole muscle.

In contrast to whole muscle, 16 h of LPS treatment on myotubes caused elevated oxygen consumption. Moreover, addition of myriocin to LPS treatment increased respiration above all other treatments (fig. 2.7).

**Discussion**

This study addresses the hypothesis that LPS, an exogenous inflammatory agent that is elevated in the blood of obese individuals, elicits effects on mitochondrial function that may contribute to the pathogenesis of many of the comorbidities associated with obesity. LPS elicits these effects via an increase in the sphingolipid ceramide. Cani, et al. observed that a four-week infusion of LPS (given at a dosage much lower than that used to induce sepsis) in conjunction with normal diet feeding, resulted in a metabolic response similar to high-fat feeding, including insulin resistance, macrophage infiltration of adipose, increased inflammatory markers in the blood, and increased liver triglyceride levels. (Cani, Amar et al. 2007) These observations prompted our own experimental design, and we injected mice daily with a sub-septic dose of
LPS to mimic the inflammatory state found in obesity.

We found that LPS treatment increased ceramide biosynthesis. This effect was observed both in LPS-injected mice as well as LPS-treated murine myotubes. LPS-injected mice had increased muscle levels of ceramides compared to vehicle-injected mice (fig. 2.1) Transcript levels of SPT 1 and 2, enzymes involved in ceramide biosynthesis, were also significantly elevated (fig. 2.2). Murine myotubes also exhibited an increase in ceramide synthesis and SPT1 transcript when treated with LPS (fig. 2.3, 3-4). These findings corroborate the results of previously published studies. Holland et al. found that activation of TLR4 with LPS or palmitate induced ceramide synthesis in muscle (Holland, Bikman et al. 2011). Schilling et al. observed that LPS and palmitate co-treatment activated TLR4 signaling via MyD88 and resulted in increased ceramide synthesis in primary macrophages (Schilling, Machkovech et al. 2013). Taken together, these data support the idea that the effects of metabolic endotoxemia could be mediated through the over accumulation of ceramides.

Additionally, we found that LPS treatment was sufficient to induce increased mitochondrial fission in myotubes, measured qualitatively by confocal imaging (fig. 2.5 B), which may be a consequence of increased Drp1 transcript levels (fig. 2.5 A). It has been previously established that bacterial sepsis increases mitochondrial dysfunction as evidenced by decreased oxidative phosphorylation (Crouser 2004, Ruggieri, Levy et al. 2010). Sun et al. found that LPS decreased expression of mitochondrial biogenesis genes and increased production of ROS in a human keratinocyte model (Sun, Zheng et al. 2014). There are, however, far fewer conclusive studies examining the effect of low-dose LPS treatment on mitochondrial fission and fusion in muscle tissue. Our observation that mitochondrial fission is induced in LPS-treated myotubes represents a novel explanation for the mitochondrial dysfunction in endotoxemia.
In addition to altered mitochondrial morphology, we observed that red gastrocnemius samples from LPS-injected mice exhibit decreased respiration compared to PBS-injected mouse tissues (fig. 2.6). These findings coincide with the observations made by Tsukumo, et al. who found that interrupting the LPS/TLR4 signaling cascade via a loss-of-function mutation of TLR4 increased oxygen consumption and decreased respiratory exchange ratio, indicating an elevation in fatty acid oxidation (Tsukumo, Carvalho-Filho et al. 2007). Frisard et al. also found that LPS treatment decreased fatty acid oxidation (measured using radiolabeled palmitate) in murine myotubes (Frisard, McMillan et al. 2010).

In all of the above-mentioned cases, we observed that inhibiting ceramide synthesis with myriocin ameliorated the effects of LPS on mitochondrial dynamics and respiration. Myriocin injections with or without concomitant LPS injections resulted in decreased ceramides in muscle tissues (fig. 2.1). The LPS-stimulated rise in myotube ceramides was also blocked by myriocin co-treatment (fig. 2.3). Taken together, these results indicate the efficacy of myriocin treatment in the ablation of de novo ceramide synthesis. This inhibition of ceramide synthesis was sufficient to prevent an observable increase in mitochondrial fission and significantly decreased fission gene transcript levels (fig. 2.5). We have previously shown that myotubes treated with ceramide or palmitate exhibit rapid alterations in the balance of mitochondrial fission and fusion, tipping the scales toward a fractionated mitochondrial state (Smith, Tippettts et al. 2013). Together, these data suggest that LPS-induced ceramide accrual was responsible for triggering the fission. Likewise, when we measured respiration in myriocin-injected animals, myriocin alone had no effect on respiration, but when given in conjunction with LPS-injections respiration was restored to levels similar to control (fig. 2.6). Again, ceramide appears to be an important mediator in the mitochondrial dysfunction observed with LPS treatment.
When we performed LPS treatment experiments *in vitro*, we expected a similar however less robust outcome on mitochondrial respiration than we had observed in tissue because of the additive effect of whole-body TLR4 activation. In contrast, LPS treated myotubes demonstrated improved respiration, and myriocin elicited an even greater respiratory response. These results counter the *in vivo* observations. We suspect this contradiction may be explained by the heterogeneous nature of skeletal muscle as compared to cultured myotubes as well as the much more complex system that is an entire organism versus a culture dish. To reconcile the differences in respiration between whole muscle and cultured myotubes, we intend to perform future co-culture studies utilizing cultured RAW 264.7 macrophages treated with LPS. We believe that it is likely that one or more other cell types are responding to the LPS *in vivo*, and these tissues are releasing signals that are affecting the myocytes. To replicate this scenario we will treat macrophages with LPS and then move the conditioned medium from the RAW cells to murine myotubes and allow them to incubate. Following said incubation, we expect to observe a blunted respiratory response as a result of the conditioned medium.

The model of LPS treatment that was used in these studies mimics the increased inflammation that is found in metabolic endotoxemia. The obese inflammatory state is partially characterized by increased LPS, however another major component of the inflammation is the presence and effects of endogenous proinflammatory cytokines that are released from adipose tissue macrophages (ATM) in obese individuals. We chose to work with LPS for the purposes of this study not only because metabolic endotoxemia is a relevant condition, but also because activation of TLR4 results in increased levels of cytokines such as TNFα and IL-6 through the MyD88 dependent pathway (Lu, Yeh et al. 2008). These cytokines are elevated as a result of ATM-induced inflammation. This study did not measure blood levels of these cytokines.
however, so it is limited in its relevance to the endogenous inflammatory state in obese individuals. Future studies examining the effect of low-dose cytokine administration would likely provide additional insights.

The rationale for this study derives from several previous observations. Firstly, LPS-triggered activation of TLR4 induces ceramide synthesis (Holland, Bikman et al. 2011), and secondly, intramuscular ceramide accrual blunts mitochondrial respiration and leads to mitochondrial fission and increased ROS production (Smith, Tippetts et al. 2013). Our additional findings that the effects of LPS treatment (increased ceramide synthesis, mitochondrial fission, and blunted mitochondrial respiration) can be prevented by inhibiting the rate-limiting step of ceramide de novo synthesis provide evidence that ceramide is a potent mediator of the effects of LPS in muscle tissue and myotubes. These results provide insight for potential therapeutic targets in individuals with symptoms of metabolic endotoxemia.

Acknowledgements

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Figure 2.1: Chronic LPS Injections Increase Ceramides in Soleus. Male C57B1 mice received intraperitoneal injections of LPS dissolved in endotoxin-free PBS (0.1 mg/kg body weight, daily), myriocin dissolved in endotoxin-free PBS (0.3 mg/kg body weight, 3x per week), both myriocin and LPS injections, or an equivalent volume of endotoxin-free PBS without LPS or myriocin (3x per week). Following the 28 day treatment period, lipids were extracted from soleus and quantified. *, $p < 0.05$ for LPS vs vehicle. #, $p < 0.05$ for LPS vs LPS+Myr. (n = 6)
Figure 2.2: Chronic LPS Injections Increase Ceramide Synthesis Enzyme Expression. Male C57B1 mice received intraperitoneal injections of LPS dissolved in endotoxin-free PBS (0.1 mg/kg body weight, daily), myriocin dissolved in endotoxin-free PBS (0.3 mg/kg body weight, 3x per week), both myriocin and LPS injections, or an equivalent volume of endotoxin-free PBS without LPS or myriocin (3x per week). Following the 28 day treatment period, SPT1 (A) and SPT2 (B) gene expression in gastrocnemius was quantified via qPCR. *, p < 0.05 for LPS vs. vehicle. (n = 6)
Figure 2.3: LPS Increases Ceramides in Myotubes. C2C12 myotubes were treated with vehicle (PBS), LPS (100 nl/ml), or LPS and myriocin (10 μM) for 16 h. Lipids were extracted and quantified. *, $p < 0.05$ for LPS vs PBS. (n = 4)
Figure 2.4: LPS Increases Ceramide Synthesis Enzyme Expression in Myotubes. C2C12 myotubes were treated with vehicle (PBS), LPS (100 nl/ml), or LPS and myriocin (10 μM) for 16 h. SPT1 expression was quantified via qPCR. (n = 2)
Figure 2.5: LPS Induces Mitochondrial Fission

A, Male C57B1 mice received intraperitoneal injections of LPS dissolved in endotoxin-free PBS (0.1 mg/kg body weight, daily), myriocin dissolved in endotoxin-free PBS (0.3 mg/kg body weight, 3x per week), both myriocin and LPS injections, or an equivalent volume of endotoxin-free PBS without LPS or myriocin (3x per week). Following the 28 day treatment period, Drp1 gene expression in gastrocnemius was quantified via qPCR. *, p < 0.05 for LPS vs vehicle. #, p < 0.05 for LPS vs LPS+Myr. (n = 6).

B, C2C12 myotubes were treated with vehicle (PBS), LPS (100 nl/ml), or LPS and myriocin (10 μM) for 16 h. MitoTracker Red was then added to visualize mitochondria via confocal microscopy.
Figure 2.6: Chronic LPS Injections Reduce Mitochondrial Respiration. Oxygen consumption was determined in permeabilized red gastrocnemius treated with vehicle (PBS), lipopolysaccharide alone (LPS), myriocin (MYR), or with LPS and myriocin (LPS+MYR). Axis labels indicate the following: glutamate+malate+succinate stimulated leak respiration (GMS\textsubscript{L}) and glutamate+malate+succinate stimulated oxidative phosphorylation (GMS\textsubscript{P}). *, p < 0.05 for LPS vs PBS. (n = 6)
Figure 2.7: LPS Increases Mitochondrial Respiration in Myotubes. Myotubes were treated with LPS (100 ng/ml) or vehicle (PBS) in the presence or absence of myriocin (10 µM) for 16 h. Following treatment, the rate of oxygen consumption was determined with SUIT1 (see Methods). Axis labels indicate the following: glutamate+malate stimulated leak respiration ($GM_L$); glutamate+malate stimulated oxidative phosphorylation ($GM_P$); glutamate+malate+succinate stimulated oxidative phosphorylation ($GMS_P$); and uncoupled respiration ($GMS_E$). *, $p < 0.05$ for treatment vs con. (n = 4)
CHAPTER 3: Mitochondrial Fission Mediates Ceramide-induced Metabolic Disruption in Skeletal Muscle

Abstract

Ceramide is a sphingolipid that serves as an important second messenger in an increasing number of stress-induced pathways. Ceramide has long been known to affect the mitochondria, altering both morphology and physiology. We sought to assess the impact of ceramide on skeletal muscle mitochondrial structure and function. A primary observation was the rapid and dramatic division of mitochondria in ceramide-treated cells. This effect is likely a result of increased Drp1 action, as ceramide increased Drp1 expression and Drp1 inhibition prevented ceramide-induced mitochondrial fission. Further, we found that ceramide treatment reduced mitochondrial O2 consumption (i.e., respiration) in cultured myotubes and permeabilized red gastrocnemius muscle fiber bundles. Ceramide treatment also increased H2O2 levels and reduced Akt/PKB phosphorylation in myotubes. However, inhibition of mitochondrial fission via Drp1 knockdown completely protected the myotubes and fiber bundles from ceramide-induced metabolic disruption, including maintained mitochondrial respiration, reduced H2O2 levels, and unaffected insulin signaling. These data suggest that the forced and sustained mitochondrial fission that results from ceramide accrual may alter metabolic function in skeletal muscle, which is a prominent site not only of energy demand (via the mitochondria), but also of ceramide accrual with weight gain.

Introduction

The current worldwide trends of increasing prevalence of obesity and type 2 diabetes suggest the complexity of the problem and the need for intensive research to discover effective therapeutic interventions. The perfect metabolic storm of dietary excess, poor nutrient quality, and reduced physical activity combine to increase the risk of developing insulin resistance,
which is not only the foundation of type 2 diabetes, but is also a contributing cause and consequence of obesity (Hotamisligil, Peraldi et al. 1996, Kimokoti, Gona et al. 2012). These causal factors (i.e., poor diet and sedentary living) lead to two distinct theories regarding the etiology of obesity-induced metabolic disruption, namely altered mitochondrial function and ceramide accumulation.

Despite being commonly represented as small, segmented structures, mitochondria are highly dynamic organelles undergoing continuous fission and fusion. Indeed, the word mitochondrion itself reveals the typical state of the mitochondrion (mitos means thread) as reticular rather than segmented. Increasingly, evidence suggests that mitochondrial structure determines mitochondrial function (Liesa, Palacin et al. 2009), including substrate metabolism (Jheng, Tsai et al. 2012, Sebastian, Hernandez-Alvarez et al. 2012) and mitochondrial bioenergetics (Westermann 2012). Given that obesity and type 2 diabetes have been considered a malady of maladaptive mitochondria, it is not surprising that interventions to stimulate mitochondrial fission or fusion elicit disparate effects on metabolic function, such as mitochondrial respiration (Westermann 2012) and insulin signaling (Jheng, Tsai et al. 2012). A consensus suggests mitochondrial fusion as beneficial in maintaining insulin sensitivity and resisting fat gain with dietary challenge (Jheng, Tsai et al. 2012, Sebastian, Hernandez-Alvarez et al. 2012).

In addition to altered mitochondrial function, the second factor that may explain obesity-induced metabolic disruption is the accumulation of the sphingolipid ceramide. Ceramide is known to accumulate in insulin-sensitive (Holland, Bikman et al. 2011) as well as high-metabolic-rate organs (Giusto, Roque et al. 1992, Lightle, Oakley et al. 2000, Holland, Bikman et al. 2011, Bikman, Guan et al. 2012), eliciting a potentially powerful impact on whole-body
metabolic function. Ceramide is known to induce insulin resistance via decayed Akt/PKB phosphorylation, as well as increase the generation of mitochondria-derived reactive oxygen species (ROS) (Kim, Choi et al. 2005, Summers 2006), which is increased with mitochondrial fission (Youle and KARBOWSKI 2005).

With these two distinct theories in mind (i.e., altered mitochondrial function and ceramide accrual), we sought to test the unifying hypothesis that ceramide forces mitochondrial fission, which is a critical mediator of ceramide-induced metabolic disruption. In support of this hypothesis, we observed that exogenous and endogenous ceramides increased mitochondrial fission in cultured cells via dynamin-related protein 1 (Drp1). Increased fission was associated with reduced mitochondrial respiration, likely at complex II, and increased production of reactive oxygen species. Moreover, we found that mitochondrial fission is important in ceramide-induced insulin resistance, as cells co-treated with a Drp1 inhibitor maintained insulin signaling despite the presence of ceramide. Lastly, mice fed a high-fat, high-sugar (HFHS) diet experienced glucose and insulin intolerance relative to standard diet-fed mice and HFHS-fed mice supplemented with myriocin to inhibit serine palmitoyltransferase, the rate-limiting enzyme in ceramide biosynthesis. Moreover, the myriocin-treated, HFHS-fed mice did not gain excess weight. Additionally, mitochondrial respiration was partially blunted in the red gastrocnemius of the HFHS-fed mice, but not those receiving myriocin. In the end, these studies suggest the potential efficacy of therapies that reduce excess ceramide accrual and/or prevent ceramide-induced mitochondrial fission in the treatment of obesity and insulin resistance.

Methods

Animals

All experiments were performed on male C57Bl/6 mice between 8 and 20 wks. 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. All animals were housed in a temperature controlled (20-21°C) environment with a 12hr:12hr light-dark cycle. Water and food were administered *ad libitum*. Mice received the following diets: 1) standard diet (SD; Harlan Teklad 8604), 2) high-fat, high-sugar diet (HFHS; Harlan Taklad 88137 + 15% (w/v) sucrose water), 3) SD with myriocin (SD+Myr), 4) HFHS with myriocin (HFHS+Myr). Myriocin supplementation was supplied in drinking water at 2.5 µg/ml, which, with average drinking patterns, equates to roughly 0.3 mg/kg/day (del Rio, Pabst et al. 2007).

**Glucose and Insulin Tolerance Tests**

Following treatment period, mice underwent i.p. glucose (Sigma-Aldrich; G7021) and insulin (Actrapid; Novo Nordisk) tolerance tests. For both tests, mice were fasted for 6 hours and received an injection of either glucose (1 g/kg body weight) or insulin (0.75 U/kg body weight). Blood glucose was determined at the times indicated in the figures, using the Bayer Contour glucose meter.

**Cell Culture**

C2C12 murine myoblast cells were maintained in DMEM plus 10% fetal bovine serum (Invitrogen). For differentiation into myotubes, C2C12 myoblasts were grown to confluency and the medium was replaced with DMEM plus 10% horse serum (Invitrogen). Myotubes were used for experiments on day 4 of differentiation.

**Cell Treatments**

For fatty acid treatment, palmitic acid (Sigma-Aldrich; catalog no. P5585) was dissolved
in ethanol and diluted to desired concentration in DMEM containing 2% (w/v) BSA (Sigma-Aldrich; A9576) and added to culture at 0.5 mM, which is in a physiological post-prandial fatty acid concentration (Fraser, Thoen et al. 1999). For ceramide treatment, C2 ceramide (N-Acetyl-D-sphingosine, Sigma-Aldrich; A7191) was diluted in ethanol in a 10 mM stock solution. For Mdivi-1 treatment, Mdivi-1 (Sigma-Aldrich; M0199) was diluted in dimethyl sulfoxide (DMSO) in a 50 mM stock solution. For LPS treatment, O55:B5 lipopolysaccharide from *E. coli* (Sigma-Aldrich; L2880) was diluted in ddH2O in a 1mg/ml stock. For myriocin treatment, myriocin (Sigma-Aldrich; M1177) was diluted in methanol in a 5mM stock solution. For sptlc2 knock down, procedures were followed according to the manufacturer instructions (Santa Cruz Biotechnology, sc-77377).

**Lipid Analysis**

For isolation of total lipids, pellets were re-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 internal standard (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 60,000 (fwhm at 400 mz) for all primary MS1 scans. MS2 fragmentation data was also collected 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 10 μL/min using a direct-inject electrospray ionization (ESI) soft-ionization spray head from a Hamilton GASTIGHT glass syringe. The spray voltage and capillary temperature were maintained at 5.0 KV and 275oC 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, Subramaniam 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.

Confocal Microscopy

C2C12 myoblasts were grown to confluence in chamber slides (NUNC Lab-Tek II Chambered Coverglass System; 155382) and differentiated at day 4. The mitochondrial dye MitoTracker Red CMXRos (Molecular Probes; M7512), dissolved in anhydrous dimethylsulfoxide (DMSO), was added to cultured myotubes at a concentration of 250 nM. The cells were incubated for 30 minutes at 37°C in the dark and then visualized using a confocal
microscope (Olympus IX81).

**Transmission Electron Microscopy**

For muscle mitochondria imaging, red gastrocnemius was extracted and fixated in 0.1M sodium cacodylate and 3% gluteraldehyde at pH 7.3 for 3 hours. The tissues were washed in 0.1M sodium cacodylate (6x, 10 min) and then washed in ddH2O (6x, 10 min). Postfixation occurred in 1% OsO4 (0.1M, 2h, 22˚C). The samples were then soaked overnight in 0.5% uranyl acetate at 4˚C. The tissues were dehydrated in a graded series of acetone (10 min, 10%, 30%, 50%, 70%, 95%, 100% 3x) then embedded in Spurrs® resin. Ultrathin sections (90nm thick) were cut with a microtome and diamond blade then stained with 0.4% lead citrate. The samples were then imaged with transmission electron microscopy.

**Quantitative Real-time PCR**

Total RNA was extracted and purified from tissues using TRIzol (Invitrogen) according to the manufacturer’s recommendations. cDNA was synthesized from mRNA via reverse-transcriptase PCR using a commercial cDNA synthesis kit with oligo(dT) primers (iScript Select cDNA Synthesis; Bio-Rad). Quantitative realtime PCR was performed with Evagreen Ssofast (Bio-Rad) using a BioRad CFX Connect Real-Time PCR Detection system. Primer sequences were 5′-ACAGGATGCAGAAGGAGATTAC and 5′-CACAGAGTACTTGCGCTCAGGA as forward and reverse primers for actin, 5′-ACTTGACCTCCCTACTGGA and 5′-TCCTCTATCCCCGTTGACACC as forward and reverse primers for Drp-1, and 5′-AAGTCCGGGAAGCTGAAAGT, 5′-TCTCGGTTATGGAACCAACC as forward and reverse primers for Mfn2. β-Actin reactions were performed side by side with every sample analyzed. Changes in mRNA level of each gene for each treatment were normalized to that of the β-actin control mRNA according to Pfaffl (Pfaffl 2001).
Protein Analysis

Tissue and cell extracts were lysed and protein content was determined using a BCA protein assay (Pierce, Rockford, IL, USA) and sample volumes were adjusted so that precisely 40 μg of protein was loaded into each lane. After addition of sample buffer, samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using methods described previously (Bikman, Zheng et al. 2010). After incubation with primary antibody, blots were incubated with a horseradish peroxidase-conjugated secondary antibody. Horseradish peroxidase activity was assessed with ECL solution (Thermo Scientific, Rockford, IL, USA) and exposed to film. Primary antibodies: SPT2 (Abcam; ab23696), pAKT (Cell Signaling; 4058S), Akt/PKB (Sigma-Aldrich; 217005), OxPhos Complex Kit (Invitrogen; 457999), Actin (Cell Signaling; 8457S). Secondary antibodies: anti-mouse (Cell Signaling; 7076S) anti-rabbit (Cell Signaling; 7074S).

Cell and Muscle Fiber Bundle Permeabilization

For cells, C2C12 myotubes 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 rpm at RT. After removal of supernatant, cells were resuspended in mitochondrial respiration buffer 05 (MiR05; 0.5 mM EGTA, 10 mM KH₂PO₄, 3 mM MgCl₂-6 H₂O, 60 mM K-lactobionate, 20 mM HEPES, 110 mM Sucrose, 1 mg/ml fatty acid free BSA, pH 7.1) plus 1 mg/ml digitonin and gently rocked at RT for 5 min before centrifugation at 1000 rpm 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). Following respiration protocol (outlined below), cells were removed from the chambers and used for further analysis, including protein quantification. For skeletal muscle use, red gastrocnemius was quickly removed from
mice following cervical dislocation and immediately placed in ice-cold buffer X (60 mM K-MES, 35 mM KCl, 7.23 mM K$_2$EGTA, 2.77 mM CaK$_2$EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM ATP, 15 mM PCr, 6.56 mM MgCl$_2$-6H$_2$O, 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 a size of around 1 mg. Bundles were then transferred 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 KH$_2$PO$_4$, 5 mM MgCl$_2$-6H$_2$O, 0.5 mg/ml BSA, pH 7.1) at 4°C for at least 15 min.

**Mitochondrial Respiration**

High-resolution O$_2$ consumption was determined at 37°C in permeabilized cells (MiR05) and fiber bundles (buffer Z) using the Oroboros O2K Oxygraph. 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 according to two protocols.

To determine general electron transport system function from red gastrocnemius tissue, electron flow through complex I+II was supported by glutamate+malate+succinate (GMS; 10, 2, and 10 mM, respectively) to determine oxygen consumption from proton leak (GMSL). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GMS$_P$). To determine full electron transport system (GMS$_E$) 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 O2 flux was reached). Complex II-supported ETS was then measured by inhibiting complex I with rotenone (Rot; 0.5 µM). Lastly, residual oxygen consumption was measured by adding antimycin
A (2.5 µM) to block complex III action, effectively stopping any electron flow. This provides a rate of respiration that is used as a baseline and all presented values are controlled for by using this baseline.

To determine general electron transport system function from murine myotubes, a substrate-uncoupler-inhibitor-titration (SUIT) protocol was used (Jheng, Tsai et al. 2012). For SUIT1, to determine general electron transport system function, electron flow through complex I was supported by glutamate+malate (GM; 10 and 2 mM, respectively) to determine oxygen consumption from proton leak (GMₐ). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GMₚ). The integrity of the outer mitochondrial membrane was then tested by adding cytochrome c (10µM; GMcP). Succinate was added (GMSₚ) for complex I+II electron flow into the Q-junction. To determine full electron transport system (GMSₑ) 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 O₂ flux was reached). Lastly, residual oxygen consumption was measured by adding antimycin A (2.5 µM) to block complex III action, effectively stopping any electron flow. This provides a rate of respiration that is used as a baseline, and all presented values are controlled for by using this baseline.

**H₂O₂ Emission**

H₂O₂ emission was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes; A22188). A reaction mixture containing 50 uM Amplex Red and 0.1 U/mL HRP in Krebs-Ringer phosphate glucose (KRPG) buffer was prepared (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl₂, 1.22 mM MgSO₄, 5.5 mM glucose). The reaction mixture was prewarmed in a 96 well plate with 100 uL of mixture per well. 20 uL
of cells suspended in KRPG buffer (~1.5x10^4) were added to each well. Samples were incubated for 1 h. Fluorescence was measured with a microplate reader (Molecular Devices; Gemini EM).

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 Increases Mitochondrial Fission Via Drp-1

Dynamin-related protein (Drp) 1 is a mitochondrial fission protein that induces outer membrane ‘pinching’ in the division of tubular mitochondria. Drp1 mRNA expression was significantly increased in C2C12 myotubes within 4 h of C2-ceramide (20 µM) treatment, and peaked after 8 h with a three-fold increase compared to baseline (fig 3.1B). Further, C2-ceramide treatment (20 µM) caused mitochondrial fission in mouse C2C12 myotubes after 16 h of treatment (fig. 3.1A). However, pretreatment with Mdivi-1, an inhibitor of Drp1 (Cassidy-Stone, Chipuk et al. 2008), prevented mitochondrial fission (fig. 3.1A). Functionally, C2-ceramide-induced mitochondrial fission led to significantly reduced complex II-supported respiration (GMSP), as determined by O2 consumption in permeabilized C2C12 myotubes in the presence of glutamate, malate, succinate, and ADP (fig. 3.2). This was completely prevented by the co-treatment of Mdivi-1 in cell culture (fig. 3.2).

To further confirm the effect of ceramide on mitochondrial fission, we explored the role of endogenously produced ceramides in response to fatty acid treatment. Accordingly, the sptlc2 gene was knocked down via siRNA (Santa Cruz Biotechnology) to prevent ceramide biosynthesis. Complete loss of SPT2 protein was determined at 120 h (fig. 3.3A) and subsequent
experiments occurred at this time. The functionality of knock down was confirmed by the observed prevention of increased ceramide accrual with palmitate treatment (PA; fig. 3.3B). Similar to ceramide treatments, palmitate resulted in mitochondrial fission (fig. 3.4A). Drp1 gene expression was elevated roughly 3-fold in the PA-treated cells, but not in SPT2 KD cells (fig. 3.4B), suggestive of the importance of ceramide in Drp1 expression. Moreover, mitochondria in cells lacking SPT2 were more fused than control cells receiving PA (fig. 3.4A). Though not as dramatic as in C2-ceramide-treated cells, PA-treatment also blunted respiration from complex-II supported oxidative phosphorylation (GMSF; fig. 3.5).

Ceramide Inhibits Complex II-Supported Mitochondrial Respiration

Given our observations of a preferentially greater inhibition of complex II-supported mitochondrial respiration with ceramide treatment, we sought to elucidate this effect more directly. Accordingly, C2C12 myotubes were treated overnight with C2-ceramide or vehicle (EtOH). While no significant difference was observed with succinate and rotenone alone to determine leak respiration (SRotL), addition of ADP to induce oxidative phosphorylation elicited a robust increase in respiration in vehicle-treated cells that was less evident in ceramide-treated cells (fig. 3.6A). The same discrepancy was observed with the addition of the uncoupler FCCP, which collapses the chemiosmotic gradient via proton transport. This change in complex II-supported respiration occurred independent of a change in complex II amount (Complex II Fp subunit; fig. 3.6B).

Inhibition of Mitochondrial Fission Prevents Reduced Mitochondrial Respiration With Ceramide Ex Vivo

Next, we sought to replicate our findings in whole muscle. Accordingly, red gastrocnemius (RG) was dissected from C57Bl6 mice and permeabilized. After permeabilization,
fiber bundles were washed and placed in oxygraph sample chambers (O2K, Oroboros Instruments) filled with 2 ml Buffer Z and vehicle (MeOH) or C16-ceramide (20 µM). Further analyses included the addition of Mdivi-1 to inhibit Drp-1-mediated mitochondrial fission. Upon placement in the chambers, fibers bundles were incubated in the buffer at 37°C for 10 min. Following this, respiration was determined according to the indicated substrate-uncoupler-inhibitor-titration (SUIT) protocol (Jheng, Tsai et al. 2012). We observed a significant increase in leak respiration (GML) in those fibers treated with Mdivi-1, though this difference did not linger with addition of ADP (fig. 3.7). We are not the first to note increased proton leak-dependent respiration with increased mitochondrial fusion (Bach, Pich et al. 2003). In contrast to Mdivi-1 treatment, ceramide-treated muscle fiber bundles exhibited a significantly blunted respiration with complex I- and II-supported respiration. No effect was observed when fiber bundles were treated with C2- or C6-ceramide (data not shown).

Mitochondrial Fission is Necessary for Ceramide-Induced Reactive Oxygen Species Generation and Insulin Resistance in Muscle

Mouse myotubes were treated with C2-ceramide or palmitate (PA) in the presence or absence of Mdivi-1 for 12 h. Following treatment, cells were washed with PBS and exposed to Amplex Red to quantify H2O2 production. While C2-ceramide alone elicited an increase in fluorescence with Amplex Red treatment, this effect was mitigated with inhibition of mitochondrial fission by Mdivi-1 co-treatment (fig. 3.8A). Considering the evidence suggesting redox-sensitive phosphorylation events in the regulation of the insulin signaling pathway (Fisher-Wellman and Neufer 2012), we explored the effect of blocking mitochondrial fission, with its subsequent reduction in H2O2 formation, on muscle insulin signaling. Inhibition of mitochondrial fission with Mdivi-1 protected Akt/PKB phosphorylation with insulin stimulation in C2-
ceramide-treated myotubes (fig. 3.8B).

**Reactive Species Generation is not Necessary for Mitochondrial Fission or Altered Mitochondrial Respiration With Palmitate and Ceramide Treatments**

To address whether ROS are necessary for the mitochondria-specific effects of both ceramide and palmitate, we replicated earlier experiments with the addition of the glutathione precursor N-acetylcysteine, an effective reducing agent known to neutralize H$_2$O$_2$ (Ozer, Parlakpinar et al. 2005, Du, Gao et al. 2009). We found that C2-treated cells that were pretreated with NAC appeared to experience a similar degree of mitochondrial fission as the C2-treatment alone (fig. 3.9). Functionally, NAC pretreatment likewise failed to prevent reduced mitochondrial respiration in ceramide and palmitate-treated myotubes (fig. 3.9A and B, respectively)

**Inhibition of Ceramide Biosynthesis Prevents Metabolic Disruption With Dietary Challenge**

8-wk old male C57Bl6 mice were separated into one of four treatment groups for 12 wk: 1) standard diet (SD), 2) high-fat, high-sugar diet (HFHS), 3) SD with myriocin (SD+Myr), 4) HFHS with myriocin (HFHS+Myr). No differences in food or water consumption were noted between the SD and SD+Myr groups and the HFHS and HFHS+Myr groups, though both the HFHS and HFHS+Myr consumed more water than the SD groups (data not shown). Marked differences in body weight were noted early in the dietary intervention, which lingered until the conclusion of the study period. Specifically, the HFHS group, but not the HFHS+Myr group, gained significantly greater body weight over the course of the study (fig. 3.10). Further, the HFHS group experienced a significant reduction in glucose and insulin tolerance, but the HFHS+Myr group exhibited no deleterious response (fig. 3.11A and B, respectively).

Due to insufficient samples of red gastrocnemius, full statistical analyses were not
performed for ceramides and Drp-1 expression in the red gastrocnemius. Thus, to determine the
effect of intervention in a similarly highly oxidative, mitochondria-rich tissue, soleus muscle was
used as a surrogate and complimentary analysis. Both muscles are commonly used in analyses of
muscle mitochondrial capacity (Baldwin, Klinkerfuss et al. 1972). Ceramides were significantly
elevated in the soleus of the HFHS group compared with the SD groups, but not in the
HFHS+Myr group (fig. 3.12B). As mentioned, statistical analysis of red gastrocnemius
ceramides (fig. 3.12A) was not possible, though a trend appears to suggest a difference exists.
Analysis of Drp-1 expression in soleus revealed a robust increase in Drp1 expression with HFHS
diet over the other groups (fig. 3.13B), suggesting a role for Drp-1 in mediating diet-induced
mitochondrial fission. A lack of tissue for RG analysis prevented statistical analysis, though the
given data suggested a similar trend in RG as noted in soleus (fig. 3.13A). Analysis of
mitochondrial O2 consumption in permeabilized muscle (RG) fiber bundles revealed a selective
reduction in respiration in the HFHS group (fig. 3.13C). Specifically, both myriocin-treated
groups had slightly higher leak respiration in the presence of glutamate-malate alone (GML).
This trend of myriocin to increase respiration continued to various degrees throughout the
protocol, but was most apparent in the HFHS group. Moreover, respiration was blunted with
both complex I- and II-mediated oxidative phosphorylation, though the difference was once
again amplified with complex II-supported respiration. A portion of each RG was prepared for
electron microscopy to determine mitochondrial morphology with the various interventions (fig.
3.14). Little difference in size of both the subsarcolemmal (left images) and intramyofibrillar
(right images) mitochondrial populations was noted between the SD-fed animals (fig. 3.14A) and
those with myriocin supplementation (fig. 3.14B). In contrast, HFHS diet appeared to result in
markedly smaller and more segmented mitochondria than the SD groups (fig. 3.14C). However,
long-term treatment of HFHS-fed animals with myriocin prevented this (fig. 3.14D). In particular, the subsarcolemmal mitochondria in the RG from the HFHS+Myr group appeared larger than those from any other group.

Discussion

The basic conclusion of this study is that division of the cellular mitochondrial network in muscle cells mediates the reduced mitochondrial respiration and loss of insulin signaling that accompanies ceramide accumulation. Given the widespread finding of skeletal muscle ceramide accumulation with weight gain (Adams, Pratipanawatr et al. 2004, Amati, Dube et al. 2011, Bikman 2012, Bikman, Guan et al. 2012), this conclusion invites a novel perspective in understanding the observations that defective mitochondrial fusion may cause, exacerbate, or is certainly related to, obesity and insulin resistance (Bach, Pich et al. 2003, Zorzano, Liesa et al. 2009, Hernandez-Alvarez, Thabit et al. 2010, Jheng, Tsai et al. 2012, Sebastian, Hernandez-Alvarez et al. 2012).

The metabolic ramifications of the physical structure of the mitochondria have appropriately received more attention in recent years and may assist in understanding metabolic disruption with obesity. Indeed, mitochondrial morphology may be relevant in the altered fuel handling evident in the muscle of obese and diabetic humans. The observation by Kelley et al. (Kelley, Goodpaster et al. 1999, Kelley and Mandarino 2000) of a “metabolic inflexibility” in obese and diabetic subjects stemmed from the inability of these subjects to oxidize glucose when provided with a glucose load. Interestingly, these same groups of subjects have been found to carry reduced proteins that drive mitochondrial fusion, namely mitofusin (Mfn) 2 (Bach, Pich et al. 2003, Bach, Naon et al. 2005, Hernandez-Alvarez, Thabit et al. 2010). A lack or overexpression of Mfn2 decreases or increases glucose oxidation, respectively (Pich, Bach et al.
Thus, the observed metabolic inflexibility with obesity/diabetes may in reality stem from
a shift towards mitochondrial fission and reduced mitochondrial fusion. While our report focuses
on Drp1, which we consistently found elevated in response to ceramide, we also explored the
effect of ceramide on Mfn2. While HFHS diet had no effect on soleus Mfn2 expression,
myriocin supplementation had a dramatic effect—Mfn2 expression was increased significantly in
both the SD+Myr and HFHS+Myr groups (supplemental fig. 3.1). It is tempting to speculate that
ceramide accumulation in skeletal muscle would force mitochondrial fission, which would
subsequently result in reduced mitochondrial capacity and function, as well as insulin resistance,
ultimately exacerbating weight gain.

Myriocin is usually administered every 24-48 h by intraperitoneal injection. Given the
efficacy of oral myriocin supplementation, our findings reveal a novel and effective delivery
method for myriocin. Regardless of the delivery, ceramide inhibition with myriocin has
consistently led to improved metabolic profile and resistance to metabolic insults, such as diet-
induced obesity. Indeed, we observed a similar resistance to the deleterious effects of dietary
challenge on glucose and insulin tolerance as that noted by the Lopaschuk group (Ussher, Koves
et al. 2010). Moreover, it is tempting to speculate that the reduced whole-body oxygen
consumption in the high-fat diet (HFD) group, but not in the myriocin treated HFD group, in
their study (Ussher, Koves et al. 2010) is partly a result of the more dramatic and disparate
oxygen consumption rates we observed in permeabilized ceramide-depleted and –replete muscle
cells and fiber bundles (figs 3.2, 3.5, 3.7, 3.13). We also noted a lack of body weight gain with
myriocin supplementation in spite of HFHS diet intervention (fig. 3.10). These results support
similar findings of an obesity-resistant effect of myriocin (Yang, Badeanlou et al. 2009) and
other inhibitors of ceramide biosynthesis (Bikman and Summers 2011), be they pharmacological
The results from our study support those by Jheng et al. (Jheng, Tsai et al. 2012) that recently explored the effect of lipid overload and dietary challenge on mitochondrial fission and function. While they did not elucidate the role of ceramides, Jheng et al. (Jheng, Tsai et al. 2012) found that palmitate, but not unsaturated oleate, increased mitochondrial fission. Interestingly, they found that addition of oleate to palmitate-treated cells prevented mitochondrial fission evident with palmitate alone. Given our previous observation that oleate prevents palmitate-induced ceramide accrual (Bikman, Guan et al. 2012), the findings by Jheng et al. (Jheng, Tsai et al. 2012) support ceramide’s role as an activator of mitochondrial fission. Further, Jheng et al. observed that Drp1 expression was increased with palmitate treatment and was an essential mediator of palmitate-induced mitochondrial fission. Critically, while we similarly found an increase in Drp1 expression with palmitate treatment, inhibition of ceramide synthesis blunted the effect (fig. 3.4B).

These studies suggest a paradigm wherein ceramide leads to altered mitochondrial dynamics, with subsequent changes in mitochondrial function, including altered respiration and reactive oxygen species (ROS) generation. However, oxidative stress from ROS is among the numerous cellular insults known to induce ceramide biosynthesis (Bikman and Summers 2011), which invites the theory that ROS are both a cause and consequence of the observed mitochondrial dysfunction. To address the possibility that ROS mediate, and thus precede, ceramide-induced changes in mitochondrial function, multiple experiments with ceramide treatment were replicated with the addition of N-acetylcysteine (NAC), a glutathione precursor known to reduce H$_2$O$_2$ (Ozer, Parlakpinar et al. 2005, Du, Gao et al. 2009, Redpath, Bou Khalil et al. 2013). Pretreatment with NAC failed to protect cells from ceramide-induced mitochondrial
fission (fig. 3.9A) as well as reduced mitochondrial respiration with both C2-ceramide and palmitate treatment (fig. 3.9B, C). Altogether, these findings suggest that ROS do not mediate the mitochondria-specific effects of ceramide accumulation.

The site of ceramide biosynthesis in the cells may be relevant in the ability of ceramide to induce mitochondrial fission. Interestingly, the two organelles involved in mitochondrial fission, namely the mitochondria and the endoplasmic reticulum (ER), are two prominent sources of intracellular ceramide (Shimeno, Soeda et al. 1998, Bionda, Portoukalian et al. 2004, Summers 2006). Physical coupling between the ER and mitochondria is necessary for mitochondrial fission, which may explain the somewhat disparate findings that have studied ceramide and mitochondrial function. Previous work that has explored the specific effect of ceramide on mitochondrial respiration has been performed largely, perhaps exclusively, in isolated mitochondria (Gudz, Tserng et al. 1997, Di Paola, Cocco et al. 2000, Yu, Novgorodov et al. 2007, Bikman and Summers 2011). Our observation of a ceramide-induced reduction in mitochondrial respiration in intact cells and muscle fibers is the first we know of. The different sources and state of the mitochondria (i.e., isolated heart mitochondria vs mitochondria in permeabilized skeletal muscle cells and fibers) may explain differences in the effects of ceramide on specific aspects of mitochondrial oxygen consumption. In particular, we consistently found decay in complex II-mediated respiration, whereas Gudz et al. (Gudz, Tserng et al. 1997) found complex III highly affected by ceramide accrual, though complex II involvement in respiration was not determined in this study. Di Paolo et al. (Di Paola, Cocco et al. 2000), in contrast, found a complex II-specific inhibition of ceramide with state 3 (ADP-supported) respiration, which corroborates our findings (fig. 3.6). It is important to note that given the necessity of physical interaction between ER and mitochondria for mitochondrial fusion and fission, isolated
mitochondria seem to be a less ideal model with which to explore the role of mitochondrial structure in altering mitochondrial respiration. Indeed, we have found that treating isolated heart mitochondria with Mdivi-1 is not effective at preventing a loss of respiration with ceramide (Tippetts and Bikman, unpublished observation). Given that ceramide reduces respiration in isolated mitochondria (Gudz, Tserng et al. 1997, Di Paola, Cocco et al. 2000), and presumably in the absence of any known fusion-fission events, these observations introduce the possibility of a fission-independent mechanism. However, we believe our consistent findings in whole cells and muscle fibers that fission is required for ceramide-induced loss of mitochondrial respiration represents the likely physiological event, where changes in mitochondrial structure can occur very rapidly. Indeed, significant mitochondrial fission can occur in minutes in response to palmitate (Jheng, Tsai et al. 2012). Perhaps related to this, we found that waiting 5-10 min was necessary to observe a reduction in respiration in muscle fiber bundles treated with C16-ceramide (fig. 3.7), which is sufficient time for ceramide to cause mitochondrial fission (supplemental fig. 3.2).

To confirm the specificity of ceramide to modify mitochondrial structure and function, we regularly used dihydroceramide as a negative control. Dihydroceramide (C2, C6, C16 in permeabilized muscle fibers and C2 in permeabilized C2C12 myotubes) had no effect on altering mitochondrial respiration, a finding supported by others (Gudz, Tserng et al. 1997, Di Paola, Cocco et al. 2000). Nevertheless, dihydroceramides, the immediate precursor to ceramides, may regulate respiration. Siddique et al. (Siddique, Li et al. 2013) recently found that cells deficient in dihydroceramide desaturase 1 (Des1-/-), which lack the enzyme that introduces the distinguishing double bond in the sphingolipid backbone, accumulate dihydro sphingolipids, including dihydroceramides, exhibit reduced mitochondrial respiration (Siddique, Li et al. 2013).
A critical difference may be the experimental protocols used in the various studies. Whereas our report and others (Gudz, Tserng et al. 1997, Di Paola, Cocco et al. 2000) that found no effect of dihydroceramides used exogenous (and mostly non-biological, e.g., C2, C6) dihydroceramides, the results by Siddique et al. (Siddique, Li et al. 2013) were observed under conditions of endogenously altered dihydrosphingolipid levels where a variety of dihydroceramides are increased, including C18, C24, and C24:1.

Ceramide has long been known to inhibit insulin signaling via a two-pronged attack on Akt/PKB action—inhibiting Akt/PKB translocation to the cell membrane as well as activating a protein phosphatase that prevents sufficient Akt/PKB phosphorylation (Salinas, Lopez-Valdaliso et al. 2000, Stratford, DeWald et al. 2001). The observation in this report, and others (Jheng, Tsai et al. 2012), of the insulin-sensitizing effect of inhibited mitochondrial fission may lend credence to the theory that redox-sensitive alterations in phosphorylation of proteins relevant in insulin signal transduction may mediate insulin resistance (Fisher-Wellman and Neufer 2012).

Inhibition of ceramide-induced mitochondrial division may have diverse clinical applications, from acutely protecting against ischemia/reperfusion injury (Novgorodov and Gudz 2009) to chronically treating insulin resistance (Jheng, Tsai et al. 2012). Ultimately, however, an erroneous assumption from these results would be that mitochondrial fusion is better for the cell than mitochondrial fission. In reality, maintaining freedom for both fusion and fission is a fundamental factor to ensure adequate bioenergetic utility of the mitochondria. Our observations suggest that ceramide may prevent the healthy dynamic cycling of mitochondria, essentially forcing sustained fission. Given the relevance of mitochondrial morphology to obesity and insulin resistance/diabetes (Zorzano, Liesa et al. 2009), these findings add to the mounting obvious applications and benefits of pharmaceutical therapies aimed at preventing excess
ceramide biosynthesis. Nonetheless, and importantly, ongoing efforts should explore the most effective combination of physical activity and, especially, improved nutritional intervention to prevent excess ceramide accumulation and alleviate subsequent metabolic disruption.

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Figure 3.1: Ceramide Induces Mitochondrial Fission.

A, C2C12 myotubes were treated with vehicle (EtOH), C2-ceramide (40 µM), or C2-ceramide and Mdivi-1 (150 µM) for 16 h. MitoTracker Red was then added to visualize mitochondria via confocal microscopy. B, Following treatment with C2-ceramide (40 µM) for the indicated time points, Drp1 gene expression was quantified via qPCR. *, p < 0.05 for C2 vs EtOH. (n = 5)
Figure 3.2: Ceramide Reduced Mitochondrial Respiration.
Myotubes were treated with C2-ceramide (40 µM) or vehicle (EtOH) in the presence or absence of Mdivi-1 (150 µM) for 16 h. Following treatment, the rate of oxygen consumption was determined with SUIT1 (see Methods). *, $p < 0.05$ for C2 vs EtOH. #, $p < 0.05$ for Mdivi vs Con. (n = 3)
Figure 3.3: SPT2 Knock-Down Prevents Palmitate Stimulated Ceramide Increase. 

A, SPT2 was knocked down via Sptlc2 siRNA in C2C12 myotubes. (n = 3) 

B, The functional effect of the SPT2 KD was confirmed by treating control (Con) and SPT2 KD myotubes. *, p < 0.05 for PA vs BSA. #, p < 0.05 for SPT2 KD vs Con. (n = 5)
Figure 3.4: Palmitate Mimics Effects of Ceramide on Mitochondrial Structure. 
A, C2C12 myotubes were treated with BSA and BSA-conjugated palmitic acid (PA; 0.5 mM) for 16 h and mitochondrial network was visualized with MitoTracker Red via confocal microscopy and compared with PA-treated SPT2 KD cells. B, Following treatment with PA, Drp1 gene expression was quantified from Con and SPT KD cells via qPCR. *, $p < 0.05$ for PA vs BSA. #, $p < 0.05$ for SPT2 KD vs Con. (n = 5)
Figure 3.5: Palmitate Mimics Effects of Ceramide on Mitochondrial Function. Con and SPT2 KD myotubes were treated with BSA alone (BSA) or BSA-conjugated palmitic acid (PA) for 16 h. Following treatment, the rate of oxygen consumption was determined with SUIT1 (see Methods). *, $p < 0.05$ for PA vs BSA. #, $p < 0.05$ for SPT2 KD vs Con.
Figure 3.6: Complex II-Mediated Mitochondrial Respiration is Adversely Affected by Ceramide Treatment.  

A, Vehicle- (EtOH) and C2-ceramide-treated (C2; 40 μM) cells were permeabilized and oxygen consumption was determined with SUIT2 (see Methods) (n = 6).  

B, Complex II Fp subunit levels were determined in C2-ceramide-treated cells over various time points as an assessment of Complex II presence in the cells. *, p < 0.05 for C2 vs EtOH. (n = 2)
Figure 3.7: Mitochondrial Fission is Requisite for Ceramide-Induced Decayed Mitochondrial Respiration.

Oxygen consumption was determined in permeabilized red gastrocnemius treated with vehicle (EtOH), vehicle with Mdivi-1 (EtOH+Mdivi) to inhibit Drp-1, C16-ceramide (C16) alone, or with Mdivi-1 (C16+Mdivi). *, p < 0.05 for C16 vs EtOH. #, p < 0.05 for Mdivi vs Con. (n = 5)
Figure 3.8: Mitochondrial Fission Inhibition Prevents Ceramide-Induced \( \text{H}_2\text{O}_2 \) Generation and Insulin Signaling.

A, C2C12 myotubes were treated with vehicle (EtOH), Mdivi-1 (Mdivi), C2-ceramide (C2), and C2 with Mdivi-1 (C2+Mdivi) followed by incubation with Amplex Red. (n = 5) B, Following 16-h treatment with C2-ceramide without (C2) or with Mdivi-1 (C2+Mdivi), cells were treated with 100 nM insulin for 10 min. Inhibition of mitochondrial fission prevented both C2-induced \( \text{H}_2\text{O}_2 \) and loss of insulin signaling. *, \( p < 0.05 \) for C2 vs EtOH. #, \( p < 0.05 \) for C2+Mdivi vs C2. (n = 3)
Figure 3.9: NAC Does Not Prevent Ceramide-Induced Mitochondrial Alterations.

A C2C12 cells were treated with vehicle (EtOH), C2 (40 µM) and C2 with NAC (5 mM; C2 + Mdivi) followed by incubation with Amplex Red (n = 2). B Myotubes were treated with vehicle (EtOH) or C2 (40 µM) in the presence or absence of NAC (5 mM) for 16 h (n = 3). C Myotubes were treated with vehicle (BSA) or PA (0.5 mM) in the presence or absence of NAC (5 mM) for 16 h (n = 3). Following both treatments in B and C the rate of O2 consumption was determined with SUIT1 (see Methods). *P < 0.05 for C2 and PA compared with EtOH and BSA.
Figure 3.10: Ceramide Inhibition Prevents Diet-Induced Weight Gain. Following 12-wk dietary intervention, mice fed a high-fat, high-sugar (HFHS) diet weight significantly more than mice fed a standard diet without (SD) or with myriocin (SD+Myr). Mice fed a HFHS diet with myriocin supplementation did not gain excess weight (HFHS+Myr) *, \( p < 0.05 \) for HFHS vs HFHS+Myr. (n=6)
Figure 3.11: Ceramide Inhibition Improves Insulin Sensitivity.
Following 12-wk dietary intervention, intraperitoneal glucose (A, 1g/kg bw) and insulin (B, 0.75 units/kg bw) tolerance tests were conducted. In every case, myriocin improved the metabolic profile. *, p < 0.05 for HFHS vs HFHS+Myr. (n=6)
Figure 3.12: Skeletal Muscle Ceramide Levels With Treatments. Ceramides were measured in red gastrocnemius (A) and soleus (B). Due to shortage of sample, statistical analysis was not possible for the red gastrocnemius (n=2-3). Ceramides were significantly elevated in the soleus with HFHS compared to the SD treatments (n=6). Though the reduction in ceramides was not significant with addition of myriocin (Myr) in the HFHS group, the trend is suggestive of an effect. *, p < 0.05 for HFHS vs SD.
Figure 3.13: Ceramide Inhibition and Glucose and Insulin Tolerance. Drp-1 expression was determined in red gastrocnemius (A) and soleus (B) from mice following a 12-wk dietary intervention. Mice fed a high-fat, high-sugar (HFHS) diet expressed significantly higher levels of Drp-1 in soleus than all other groups. 

C, Oxygen consumption was determined in permeabilized red gastrocnemius from the four dietary interventions. *, p < 0.05 for HFHS vs SD. #, p < 0.05 for HFHS+Myr vs HFHS. (n=6)
Figure 3.14: Ceramide Inhibition Prevents Diet-Induced Alterations in Mitochondrial Morphology. Visualization of subsarcolemmal (left figures) and intramyofibrillar (right figures) red gastrocnemius mitochondrial fractions suggests that myriocin supplementation increases mitochondrial size ($B$ vs $A$, $D$ vs $C$) ($n=3$). In particular, HFHS ($C$) intervention appeared to result in markedly smaller mitochondria ($C$), but not with myriocin supplementation ($D$).
Supplemental Figure 3.1. Myriocin Increases Mfn2 Expression.
Mfn2 expression was determined in soleus from mice following a 12-wk dietary intervention. Mice receiving myriocin on standard diet (SD+M) and high-fat, high-sugar diet (HFHS+M) had significantly higher levels of Mfn2 than groups not receiving myriocin (SD and HFHS alone). *, p < 0.05 for myriocin treatment over vehicle treatment.
Supplemental Figure 3.2: C2-Ceramide Induces Rapid Mitochondrial Fission.
C2C12 cells were treated with vehicle (EtOH) or C2-ceramide (40µM) for 10 min prior to MitoTracker Red addition and visualized via confocal microscopy.
CHAPTER 4: Conclusion

The overarching goals of this dissertation were to determine the role of ceramide as a mediator of LPS induced altered mitochondrial function in both murine myotubes and in an \textit{in vivo} mouse model of chronic sub-clinical inflammation, as well as to determine the impact of both ceramide accumulation and ceramide depletion on metabolic rate. These studies helped provide understanding as to ceramide’s effect on muscle and whole-body metabolic function. Specifically, the following questions were addressed:

1) Is ceramide is necessary for inflammation-induced altered mitochondrial respiration?

2) Does the inhibition of \textit{de novo} ceramide synthesis reduce or eliminate the deleterious effect of LPS on mitochondrial respiration and mitochondrial dynamics?

3) Does ceramide accumulation in skeletal muscle modulate the composition and actions of proteins involved in mitochondrial form and structure?

4) Does the accumulation of ceramide in skeletal muscle reduce energy utilization by the muscle and the whole-body by regulating mitochondrial function?

We had hypothesized that ceramide accumulation in muscle tissue would result in impaired mitochondrial dynamics and function. We further hypothesized that ceramide was a necessary mediator in the metabolic dysfunction observed in inflammatory states. The resulting studies provided the following answers to the above-mentioned questions:

\textit{Ceramide is Necessary for Inflammation-Induced Altered Mitochondrial Respiration}

In support of our hypothesis, we found that gastrocnemius taken from LPS-injected mice had lower respiratory rates than vehicle-injected mice. These mice had significantly increased
levels of ceramide in their soleus muscles and increased gene transcript levels of two isoforms of SPT, the rate-limiting enzyme of ceramide synthesis.

In an unexpected turn of events, we observed that murine myotubes experience an enhancement of respiration when treated with LPS. Myriocin co-treatment amplifies this increase. Although we do not presently have any data to provide an explanation for this increase, we intend to perform future co-culture studies utilizing cultured RAW 264.7 macrophages treated with LPS. Because of the heterogeneous nature of skeletal muscle, we believe that it is likely that one or more other cell types are responding to the LPS in vivo, and these tissues are releasing signals that are affecting the myocytes. To replicate this scenario, we will treat macrophages with LPS for 24 hours. Following that treatment period, we will move the conditioned medium from the RAW cells to murine myotubes and allow them to incubate for 24 hours. Following said incubation, we will measure the respiration of the myotubes. We expect to observe a blunted respiratory response as a result of the conditioned medium.

The Inhibition of De Novo Ceramide Synthesis Reduces or Eliminates the Deleterious Effect of LPS on Mitochondrial Respiration and Mitochondrial Dynamics

As stated above, LPS injections decreased mitochondrial respiration. Mice that were injected with myriocin alone had no significant change in respiration. Animals who received both LPS and myriocin injections were protected from the decrease in respiration. Myriocin also had a protective effect against ceramide accumulation within the muscle tissue; myriocin/LPS-injected animals did not exhibit the large increase in ceramide levels in the soleus. Because ceramide synthesis inhibition was sufficient to prevent a blunted respiratory rate, we concluded that ceramide was an important mediator in the inflammation-induced alteration of mitochondrial respiration.

Animals who received LPS injections had increased transcript levels of Drp1, a potent
stimulator of mitochondrial fission. Myotubes treated with LPS and observed with a confocal microscope showed a marked increase in mitochondrial fission compared to control cells, but in cells that received a co-treatment of LPS and myriocin, excessive fission was prevented. This decrease in fission can likely be attributed to alterations in fission/fusion proteins. Gastrocnemius from LPS-injected mice showed increased gene transcript levels of Drp1, however mice receiving both LPS and myriocin injections did not demonstrate a significant increase in Drp1 expression.

Ceramide Accumulation in Skeletal Muscle Modulates the Composition and Actions of Proteins Involved in Mitochondrial Form and Structure

In support of our hypothesis, we found that ceramide treatment of murine myotubes increased transcript levels of Drp1 and increased mitochondrial fission as observed by confocal microscopy. This effect could be prevented by co-treatment with MDivi-1, a known inhibitor of Drp1, indicating that the fission-inducing action of ceramides is indeed Drp1 mediated. A similar effect on mitochondrial fission was observed when palmitate was added to cell growth medium. Inhibiting SPT2 using siRNA was sufficient to eliminate the increase in Drp1 transcription levels and prevent mitochondrial fission.

Tissue extracted from mice fed a high-fat high-sugar (HFHS) diet had significantly increased levels of ceramides in soleus tissue. Ceramides were decreased, although not significantly, in response to myriocin treatment. Transcript levels of Drp1 were increased significantly in soleus, indicating a shift toward mitochondrial fission. TEM images of HFHS diet fed mouse gastrocnemius show decrease in size of subsarcolemmal and intramyofibrillar mitochondria. Myriocin co-treatment resulted not only in rescue of the mitochondrial phenotype, but also in enlargement of the mitochondria even when compared to standard diet mouse tissue. Thus ceramides appear to be inducing mitochondrial fission in vitro as well as in vivo.
The Accumulation of Ceramide in Skeletal Muscle Reduces Energy Utilization by the Muscle and the Whole Body by Regulating Mitochondrial Function

Treatment of murine myotubes with ceramide resulted in a blunting of mitochondrial respiration. This effect was eliminated by co-treatment of MDivi-1, indicating that the ceramide-mediated decrease in respiration was mitochondrial-fission dependent. Palmitate treatment induced an effect similar to that of ceramide that could be blocked by siRNA inhibition of SPT2, the enzyme that converts palmitate to ceramide. Ceramide treatment of cells also increased ROS production and decreased Akt phosphorylation. Both of these outcomes were prevented by MDivi-1 co-treatment.

HFHS diet mice that received myriocin treatments were protected from diet-induced weight gain and maintained glucose and insulin sensitivity compared to mice without myriocin treatments. Gastrocnemius tissue from HFHS diet mice exhibited decreased levels of respiration, however HFHS diet mice that also received myriocin treatment did not. Taken together, all these data indicate that ceramide accumulation impairs substrate utilization and mitochondrial function.

Conclusion

In conclusion, LPS treatment up-regulates ceramide synthesis by increasing transcription of enzymes involved in the de novo ceramide synthesis pathway. Additionally, LPS treatment induces mitochondrial fission and blunted respiration. These effects are mitigated by inhibition of ceramide synthesis. Ceramide increases transcription of Drp1 and increases ROS production leading to mitochondrial fission, decreased Akt phosphorylation and decreased respiration. HFHS dietary challenge increases intramuscular ceramides in mice, which leads to mitochondrial fission, glucose and insulin intolerance, and impaired respiration. These data provide the insight that ceramide synthesis inhibition may be a novel target for preventing inflammation and
lipotoxic-induced metabolic dysfunction.
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PUBLICATIONS


MEETING ABSTRACTS


