Ceramides are a group of lipids in the sphingolipid family that are potent cell signaling molecules. Elevations in ceramide levels above the norm generally lead to apoptosis. Evidence from \textit{in vitro} studies suggests that accumulation of ceramides within mitochondria leads to dysfunction of the mitochondria. This includes inhibition of the electron transport chain and release of reactive oxygen species (ROS). Large elevations of mitochondrial ceramide may also lead to death of the cell as they promote membrane permeability transition, which allows the release of cytochrome c and other apoptogenic factors. With age, cardiac mitochondria show a similar dysfunctional phenotype to that found in conditions of acute ceramide elevation. We therefore hypothesize that ceramides may be accumulating in cardiac mitochondria from aged animals.
To elucidate the characteristics of the sphingolipidome, we developed both mitochondrial isolation techniques and tandem mass spectrometry assays to specifically and sensitively monitor mitochondrial sphingolipids. Using these techniques, it was found that mitochondria contain six distinct ceramide species with highly saturated lipid moieties. Using young (3-6 months old; which corresponds to a post-adolescent human) and old (24 to 28 months old; which corresponds to an elderly human) Fischer 344 rat hearts, we found that aging leads to a significant increase in total mitochondrial ceramides (32%, p < 0.03), with C_{16}, C_{18}, and C_{24:1}-ceramides showing the largest percent increases (72.3%, 73.4%, and 77.7%, respectively, p < 0.05). Furthermore, the age-associated elevation in ceramide levels correlated to a 28% decrease in the activity of complex IV of the electron transport chain (p < 0.05), which could be replicated in vitro by inducing a ceramide accumulation in mitochondria isolated from young animals.

Mitochondria do not contain enzymes for de novo ceramide biosynthesis, rather, these organelles have sphingomyelinases, a family of enzymes that cleave the phosphorylcholine headgroup from nascent pools of sphingomyelin. Specifically, mitochondria contain the magnesium-requiring isoform of sphingomyelinase with a neutral pH optima (nSMase). Recent work has shown that nSMase activity is inversely regulated by glutathione status. Because cardiac mitochondrial glutathione (mGSH) declines by up to 60% with age, we hypothesized that the loss in mGSH leads to an increase in ceramides through the upregulation of nSMase.
To determine whether loss of mGSH plays a role in the regulation of mitochondrial nSMase activity, mGSH levels were depleted by treating freshly isolated hepatocytes with 3-hydroxy-4-pentenoate (3HP). It was found that 3HP rapidly depleted mGSH in a concentration-dependent manner (EC$_{50}$ = 232 µM, p < 0.05). Moreover, this depletion led to an increase in nSMase activity (24 ± 3% at 250 µM 3HP, p < 0.05), and an increase in total ceramide levels (27%, p < 0.05). These findings suggest that mGSH status plays a critical role in the maintenance of ceramide levels within mitochondria. Furthermore, because nSMase activity is regulated by mGSH levels, we hypothesized that any agent promoting an increase in mGSH would reverse the ceramide accumulation seen in cardiac mitochondria from aged animals.

Lipoic acid (LA) is a naturally occurring dithiol compound used for many years as an anti-inflammatory agent. LA-supplementation has been shown to increase cellular and mGSH by increasing cellular cysteine levels in the aging heart. In order to determine whether LA reverses the age-associated ceramidosis in cardiac mitochondria, young and old F344 rats were pair-fed LA [0.2% (w/w) in the diet] against controls for two weeks and cardiac mitochondria were subsequently isolated and analyzed. It was found that LA-treatment reversed the age-associated decline in mGSH levels [decreased 43% with age (p < 0.05)], and reduced nSMase activity [increased 103% with age (p < 0.05)]. Ceramide levels were reduced [elevated 32% with age (p < 0.03)] so that they were no longer different from young controls and complex IV activity restored t youthful levels [declined 28% with age  (p < 0.05)].
In conclusion, this dissertation provides evidence to support a new mechanism that explains, at least in part, the progression of mitochondrial dysfunction in the aging heart, and may also contribute to understanding the age-related loss of cardiomyocytes. It also provides mechanistic insights into the overall health benefits of LA supplementation and supports its use as a safe, natural, and “age-essential” micronutrient.
A Role for Ceramide Accumulation in Age-related Cardiac Mitochondrial Dysfunction

by

Jeffrey S. Monette

A DISSERTATION

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Jeffrey S. Monette, Author
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Chapter 1

General Introduction
1.1 Background and significance

*The aging “epidemic”*

Due to advances in health care, people in the United States are living substantially longer than ever before. This, compounded with the “Baby Boomers” reaching their retirement years, has made the elderly (> 65 years old) the fastest growing section of the U.S. population (1). This age-group is estimated to expand from the current 12.8% of the U.S. population to 18.5% by 2025, which more than doubles its current population. With this increase in the elderly population comes some difficult obstacles for the medical community to overcome, as aging is a key risk factor for many diseases such as, cancer (2), arthritis (3), osteoporosis (4), type 2 diabetes (5), and Alzheimer's (6). The prevalence of these disorders in the elderly, most likely, stems from the progressive decline in organ function that characterizes the aging process.

*Aging and the heart*

Cardiac pump function substantially declines during the aging process. This loss is characterized by many deleterious structural and biochemical alterations. Diastolic function is compromised by calcification and scarring of the valves, as well as the loss of sinoatrial node cells (10% left by age 70) and increased fat and collagen deposition (7). In particular, left ventricular function, which comprises the major blood pumping capacity, declines with age. The left ventricle becomes hypertrophic and loses compliance due to myocyte loss and
restructuring of the sarcoplasm. This age-related loss in myocytes is quite substantial: myocyte number declines in men at a rate of $45/10^6$ cells per year from age 19-49. The rate of myocyte loss actually accelerates to $131/10^6$ cells per year by age 72 (8). Thus, elderly men have lost 35-50% of their cardiomyocytes. Compensatory mechanisms, such as remodeling of the sarcomeres and decreased vascular dilation, only partially mitigates the myocyte loss and ultimately results in inadequate blood pumping capacity, leading to heart failure. This represents a major burden on our health care system, as currently, up to 10% of people over the age of 65 suffer from recurring heart failure (HF). With the rapidly increasing population of elder Americans, the United States is set to see a dramatic increase in the amount of people living with HF. To treat, delay, or prevent the onset of the decline of heart function, we must first understand, mechanistically, the leading cause(s) of such deterioration.

Aside from physiological loss, biochemical deficits are also hallmarks of the aging heart. Deficiencies in calcium handling by the sarcoplasmic reticulum lead to cardiac arrhythmias (9). Energy metabolism is also altered so that glycolysis supplants fatty acid oxidation as the primary source of energy for the myocytes. This may be due, in part, to alterations in mitochondria that result in less efficient lipid handling, and inhibition of the mitochondrial electron transport chain (10-12). With age, there is an increase in the formation of reactive oxygen species (ROS), decreased antioxidants and antioxidant responses with concomitant increased oxidative damage to lipids, proteins, and DNA. As a whole, this leads to increased incidents of apoptosis, which overcomes the ability
of this post-mitotic tissue to replace damaged and dying cells (8). The loss in cells leads to hypertrophy and weakening of the heart, leaving the elderly population susceptible to many acute and chronic pathologies. The root-cause(s) of these age-related deficiencies are not well understood. However, the fact that many of the age-related deficiencies are centered on the primary functions of mitochondria (i.e. energy metabolism, ROS formation, calcium handling, and induction of apoptosis) would suggest that it is very likely that mitochondrial dysfunction plays an active role in the age-related decline in cardiac function.

The functions of mitochondria

Most of the energy needed for cellular metabolism is supplied by the mitochondria and stored as the high-energy phosphate bonds in the ATP molecule. Mitochondria utilize pyruvate and NADH (via the malate:aspartate shuttle) from glycolysis, and are the primary site where fatty acids are oxidized. Obviously, any alteration of energy metabolism can be detrimental to cell health. While mitochondria are best known for their role as the “powerhouse” of the cell, they actually perform multiple vital cellular functions. Mitochondria are the “gatekeepers” of the intrinsic pathway of apoptosis. Cell death signals such as UV damage and the unfolded protein response in the endoplasmic reticulum (13) activate release of apoptogenic factors, such as cytochrome c, from the intermembrane space of mitochondria into the cytosol. This initiates a series of events termed “the caspase cascade” that ultimately leads to apoptosis.
Furthermore, mitochondria are also linked to the extrinsic pathway of apoptosis. For example, activation of tumor necrosis factor alpha receptor leads to cleavage of caspase 8 followed by truncation of Bid to tBid. This protein then activates the pro-apoptotic members of the Bcl-2 family (i.e. BAK and BAX), which results in the release of cytochrome c and SMAC/DIABLO. Once these factors are released from mitochondria, they form the apoptosome and further initiate the caspase cleavage cascade (14).

Mitochondria are also one of the key sites for the generation of ROS (15). Damage or alterations to the electron transport chain (ETC) leads to the transfer of electrons to oxygen, which results in formation of superoxide (O$_2^-$). This radical actually has more reducing potential than most ROS; however, O$_2^-$ can form deleterious ROS (i.e. hydroxyl radicals, hydrogen peroxide, and peroxynitrite). The cell has evolved defense mechanisms to limit the damage caused by the release of these agents. Antioxidant defenses include enzymes such as superoxide dismutase (SOD) and catalase, as well as non-enzymatic antioxidants such as glutathione (GSH), protein thiols, and ascorbic acid. In normal conditions, the antioxidant system rapidly inactivates these reactive species and prevents damage to the cell.

Because of their central position in so many aspects of cellular metabolism, mitochondrial dysfunction can be extremely detrimental. In fact, mitochondrial dysfunction play a key role in many pathological states, including neurodegenerative disorders (Parkinson’s and amyotrophic lateral sclerosis [ALS]), as well as a host of pro-inflammatory conditions such as ethanol-induced
liver disease, diabetes, and atherosclerosis (10, 16-26). In organ that requires a constant supply of energy, such as the heart, mitochondrial dysfunction could have profound consequences to organ function.

Age-related dysfunction in cardiac mitochondria

In the heart, there are two subpopulations of mitochondria. Interfibrillar mitochondria (IFM) are intercalated among the myofibrils and provide the ATP needed for contraction of the muscle, whereas subsarcolemmal mitochondria (SSM) lie just below the plasma membrane and provide energy for calcium homeostasis. These two populations are functionally different. IFM have a higher oxidative capacity and utilize fuels much quicker than SSM (27). This difference is most likely due to the differing roles these two populations have in the heart.

It is generally accepted that during the aging process mitochondria undergo a systematic decline in overall function (10, 16-23). In post mitotic tissue such as the heart, this decline is perplexing due to the fact that mitochondrial pools are renewed approximately every 10 days (28, 29). With age, IFM become predominantly dysfunctional as compared to SSM. Oxidative capacity is significantly reduced in IFM (30), the activities of electron transport complexes III (31) and IV (18) decline, increased levels of oxidative damage markers are found, coinciding with an increase in antioxidants (i.e. SOD and GPX), suggesting that these mitochondria were under a state of inflammation (17). With
age, there is a marked decrease in antioxidant response leading to an increase in oxidative damage to lipids, proteins, and DNA (18).

Much research has gone into understanding the basic biochemical mechanism(s) that leads to age-related mitochondrial dysfunction, although the answer has remained elusive. Interestingly, mitochondria from aged animals share a similar phenotype as seen in pro-inflammatory pathologies, including increased ROS formation, oxidative damage, impaired electron transport, and altered energy metabolism (10, 16-22). The hypothesis that mitochondria play a significant role in the process of aging is not novel. In fact, Harman and colleagues hypothesized that mitochondrial dysfunction is not only a major factor in the onset of aging but is the cause of aging (32). While the dysfunctional phenotype of mitochondria has been known for many years, the root-cause(s) of this dysfunction is an area of current debate.

Ceramide

With respect to the mechanism(s) for the progression of the aging phenotype in mitochondria, much research effort has been put into studying mitochondrial DNA mutations, post-translational protein modifications, altered protein expression, mitochondrial biogenesis and degradation. However, a true cause-and-effect relationship has not yet been established. My research is directed towards understanding the role of the cardiac mitochondrial lipidome in the process of aging. My project focused primarily on the inner mitochondrial
membrane, as many of the deleterious alterations of mitochondrial function are related to the composition of this lipid domain (10, 16, 31, 33-36). Specifically, we wanted explore the possibility that a unique lipid, ceramide, could play a part in the modification of the inner mitochondrial membrane with age.

Ceramides are a family of lipids composed of a sphingolipid backbone with an N-acylated fatty acid residue of varying chain length (16 to 24 carbons in the heart) (Figure 1.1). They are a normal, yet minor, component of many membranes including those of the plasma membrane and lysosomes (37-40). In these organelles, ceramides are maintained at consistent concentrations, which suggests a high level of regulation. This is undoubtedly due to their potent effect on cell signaling with even a modest localized accumulation (41).

Interest in the role of ceramide accumulation to human health arose from the observation that these particular lipids tend to accumulate in tissues. This occurs during both acute and chronic diseases, especially pro-inflammatory pathologies. Disease states involving aberrant ceramide metabolism include type II diabetes (42-45), Alzheimer’s disease (46-49), Wilson’s disease (50), and cardiovascular diseases (CVD) (51-56). In atherogenesis, ceramide accumulation is linked to aggregation of LDL, increased ROS, and promotion of foam cell formation (51-56). Interestingly, Yi et al. showed that when rat mesangial cells were incubated with homocysteine, a common marker for CVD, there was a 47% increase in ceramides and increased ROS formation. Conversely, inhibition of ceramide synthesis blocked both ceramide accumulation and ROS formation (57). For diabetes, Levin et al. showed a correlation of ceramide increase with insulin
sensitivity. In his study, mice overexpressing DGAT, an acyl transferase involved in triglyceride formation, resulted in a 63% increase in type-II muscle ceramide levels and was accompanied by impaired glucose handling (43). Furthermore, evidence by Straczkowski et al. indicates that men at risk for diabetes have a 50 to 200% increase in ceramide levels within type II muscles (45). While correlative, these examples highlight the potential negative consequences of, even slight, accumulations of ceramide to human health.

Because ceramide plays a role in so many pathologies, significant effort has been put into understanding the biochemical mechanism(s) of ceramide action on cellular processes. To this end, in vitro studies show that ceramide affect cellular signaling networks, where increases in ceramide levels induce multiple signaling kinases. For example, kinase suppressor of RAS (CAPK) is a ceramide-dependent enzyme which can inhibit cell proliferation via truncating RAS-mediated cell mitosis (58). Increases in ceramide levels also induce PKCζ, the Jun-N-terminal kinases (JNKs), lysosomal cathepsin D, and phospholipase A₂ (59-62). Ceramide-mediated activation of these proteins leads to increased growth arrest and/or necrosis.

In addition to activating growth inhibitory kinases, accumulation of ceramides activate phosphatases that inhibit pro-survival signaling networks (63-65). Ceramide overload inhibits pro-survival pathways such as Akt and c-jun by activating protein phosphatase 2A (PP2A) (63, 66). PP2A, also known as ceramide-activated protein phosphatase, not only works to dephosphorylate Akt at serine 473 (which inactivates the enzyme) but also regulates the Bcl-2 protein
Figure 1.1 Structure of ceramide. Ceramides are sphingolipids synthesized in the ER and, to a lesser extent, in mitochondria. Individual ceramide species are obtained by the $N$-acylation of the sphingosine backbone with a fatty acid of a particular chain length (e.g. palmitic acid as depicted).
phosphorylation state. This latter function of PP2A infers that ceramide levels strongly influence the apoptotic threshold of cells. In fact, ceramide accumulation is an early event in induction of apoptosis following addition of many stress-inducing agents, such as tumor necrosis factor alpha (TNF-α), H₂O₂, interleukins, daunorubicin, UV light, and heat shock (58, 67-73). By activating “pro-death/growth arrest” signaling networks, while inhibiting pro-survival signaling, ceramides accumulation “tips the balance” towards growth arrest and cell death. Hence ceramide has been termed a “pro-apoptotic sphingolipid”.

**Ceramide and mitochondria**

While much is known concerning the role of ceramides in cellular signaling, there is a dearth of knowledge concerning the role ceramide plays in mitochondrial function. Although limited, initial evidence indicates that ceramide accumulation in mitochondria induce a dysfunctional phenotype that is reminiscent of that seen in aging and chronic-inflammatory pathologies (See Figure 1.2). *In vitro* studies indicate that modulation of ceramide levels dramatically affects mitochondrial function. Treatment of both mitoplasts and intact mitochondria with aqueous-soluble ceramide analogs show that acute ceramide accumulation inhibits electron transport (74, 75) and induces ROS formation from the ETC. Gudz *et al.* found ceramide inhibits Complex III of the ETC in partially purified mitochondrial extracts (74), whereas Di Paola *et al.* observed that both Complex I and IV activities were increasingly attenuated with
### Figure 1.2 Acute ceramide accumulation mimics the aged heart mitochondrial phenotype

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<tr>
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<tr>
<td>Inhibition of Complex III/IV</td>
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<tr>
<td>Increased ROS</td>
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<td>Altered Membrane Fluidity</td>
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increasing ceramide levels (76). These effects, of course, would be due to ceramide action associated with the inner mitochondrial membrane. Increased ceramide levels in the outer mitochondrial membrane would not be expected to affect the ETC, but may nevertheless induce adverse consequences to mitochondrial and cell function. Various groups showed that when ceramide levels are elevated in outer mitochondrial membranes, a remodeling of Bcl-2 family heterodimers occurs which is followed by induction of membrane permeability transition, and subsequent release of cytochrome c and other apoptogenic factors (77, 78). These observations fit with those showing ceramide induction of PP2A, as this phosphatase is known to significantly lower anti-apoptotic Bcl action through dephosphorylation of these proteins (63, 64, 78, 79).

In summary, there is limited but important evidence that ceramide accumulations in mitochondrial membranes inhibit electron transport, induce ROS and lower the anti-apoptotic threshold.

Even though the exact mechanism(s) by which ceramide influences cell signaling is still under intense investigation, it is clear that even small changes in ceramide levels potently affect cell signaling pathways. Thus, steady-state ceramide levels are strictly regulated. To complicate matters further, there is now evidence suggesting that chain length and the degree of unsaturation of the N-acyl side-chain are important for specific ceramide action in cells. Ceramides that contain either palmitic or stearic acids (C16- and C18-ceramide, respectively) particularly accumulate during pro-inflammatory conditions and specifically promote apoptosis (41, 80, 81). This concept of specific functions for a particular
ceramide species was recently strengthened by Senkal et al. who showed that squamous cell carcinomas were specifically killed by an increase in C_{18}-ceramide induced by chemotherapy (81). However, C_{18}-ceramide homologs containing even one site of unsaturation do not promote apoptosis. Despite this evidence, the potential for a particular ceramide species to affect specific cellular functions (described above) is not well understood. This is because most studies to date have used imprecise quantification methods that only monitor general ceramide levels, not the precise levels of individual ceramide species. Thus, significant work is still necessary to distinguish an exact structure/function relationship with individual ceramide species versus general accumulation of this sphingolipid.

**Regulation of ceramide levels by glutathione**

In Chapter 3, we show that there is an age-related accumulation of cardiac mitochondrial ceramides. Furthermore, this accumulation occurs in ceramide species found predominantly in the inner mitochondrial membrane. To yield a useful insight into the progression of aging, we sought to understand the mechanism(s) by which these ceramides could be accumulating. As ceramides are at a central point in the metabolism of all sphingolipids (Figure 1.3) this is not a straight-forward question. Ceramide formation occurs through four principal routes: 1) *de novo* synthesis (82), 2) enzymatic hydrolysis of native sphingomyelin pools by sphingomyelinases (82, 83), 3) re-acylation of sphingosine by reverse ceramidase action (84), 4) hydrolysis of the sugar moieties on complex sphingolipids by glucosidases (85).
De novo ceramide synthesis begins with the formation of the sphingosine backbone by the condensation of palmitoyl-CoA and serine. This is followed by reduction of the ketone and acylation of sphingosine with a fatty acid of varying chain length, which yields dihydroceramide. Ceramide is finally formed by a dehydration reaction between the carbons 4 and 5 of the sphingoid backbone (Figure 1.3, orange box). Given the extreme hydrophobicity of this sphingolipid, ceramide must be transported from sites of de novo synthesis to target membranes. Ceramide transport from its site(s) of synthesis to peripheral membranes is not well understood, but there is growing evidence that both vesicular trafficking and transport by specialized proteins (CERT proteins) are involved (86). Aside from de novo synthesis, all other routes are considered salvage pathways, as they create ceramide within target membranes from native sphingolipids (Figure 1.3, red arrows).

In general, mitochondria do not contain the necessary machinery for de novo synthesis. Some evidence exists that the mitochondrial associated membranes of the ER contain some of the synthetic machinery (87), but these membranes contain large amounts of ER. However, more highly purified mitochondrial extracts have not been shown to contain this machinery. In addition, we have preliminary evidence showing that mitochondria cannot form a sphingosine backbone from palmitate (data not shown). Overall, this suggests that the de novo synthesis route could not supply ceramides found in acute stress responses. Additionally, our results in Chapter 3 show that mitochondria do not
Figure 1.3 Sphingolipid metabolism. De novo synthesis of ceramides (in orange) occurs in the endoplasmic reticulum, further processing of these lipids occurs in the golgi apparatus followed by transport to target membranes. Ceramides can be created directly within membranes, such as the plasma membrane, through salvage pathways (red arrows).
contain significant levels of sphingosine which makes this salvage pathway equally unlikely. In addition, no report to date has confirmed the presence of a native pool of cerebrosides or gangliosides to supply substrates for their salvage pathway. On the other hand, mitochondria do contain the magnesium-requiring isoform of sphingomyelinase with a neutral pH optima (nSMase) (88). Mitochondria do not contain either the synthetic machinery or the proper concentration of substrates for de novo synthesis or utilization of the ceramidase and glucosidase salvage pathway, but do contain high levels of sphingomyelin and nSMase. This leaves the possibility that the accumulation of mitochondrial ceramide seen in aging and other pro-inflammatory disease states is caused by a disregulation of nSMase.

Further support of the pro-inflammatory nature of nSMase regulation comes from recent evidence showing that nSMase is regulated by glutathione (GSH) (89, 90). GSH is a tripeptide, γ-L-glutamyl-L-cysteinylglycine, and the primary low molecular weight antioxidant in the cell and is responsible for the maintenance of cellular thiol redox state, termination of certain free radicals, detoxification of xenobiotics, and also maintains antioxidants such as ascorbate and α-tocopherol (vitamin E) in their reduced states (91-94). In addition to these important roles, GSH alters enzymatic activity of proteins by reducing or oxidizing cysteine residues (90, 95-101). Hannun and colleagues showed that nSMase activity is inhibited by physiological concentrations (1 to 10 mM) of GSH (89, 90). The exact mechanism by which GSH regulates nSMase activity is currently unknown. However, GSH acts as a non-competitive reversible inhibitor
of nSMase (102). Furthermore, this interaction is not dependent on the thiol moiety or the redox state, but requires, at minimum, the γ-glutamyl-cysteine portion of GSH or GSSG (90). Given the role that GSH plays in the inhibition of nSMase, any decrease in GSH would lead to an increase in nSMase activity, and subsequently and increase in ceramides. In aging, cardiac mGSH content decreases by up to 60% (89, 103). This suggests that cardiac mitochondria from aged animals may undergo a GSH-dependent up-regulation of nSMase activity. This mechanism of action may explain, at least in part, the link between the decrease in antioxidant response capabilities and the actual dysfunctional phenotype of mitochondria seen with age.

The anti-inflammatory agent (R)-alpha lipoic acid

In chapter 5, we discuss the potential benefits of supplementation with (R)-alpha-lipoic acid (LA) for mitochondrial function in the aging heart. LA is a naturally occurring dithiol found in the mitochondria where it is an essential cofactor for α-ketoacid dehydrogenases (104). LA has a chiral center allowing for two enantiomers, R- and S-LA. The R-form is the naturally occurring form, but commercial LA supplements are generally a racemic mixture. The activity of LA is centered on its highly reactive vicinal thiol groups which allows LA to form a strong redox couple ($\Delta E^o = -320mV$) between its reduced (dihydrolipoate) and oxidized states.
LA has been used for decades as a dietary supplement and treatment of a range of pro-inflammatory pathologies such as diabetes (105, 106), atherosclerosis (107), multiple sclerosis (108), Alzheimer’s (109), cardiac ischemia/reperfusion injury (110), and aging (111, 112). The mechanism of LA action is still an area of debate. LA is thought to function as a potent antioxidant (107, 113), a metal chelating agent, and an inducer of anti-inflammatory signaling pathways. However, the extremely low level and transient accumulation of LA following its supplementation suggests that its dramatic effects on cellular antioxidant status could not be achieved through direct antioxidant-LA interaction. On the other hand, the ability of LA to restore proper antioxidant levels can be explained by its ability to up-regulate Nrf2-mediated transcription of anti-oxidant response genes (114).

In mitochondria from aged animals, LA-treatment lowers indices of mitochondrial dysfunction (115-117). This includes the restoration of GSH status to that seen in young animals (112). This gives us the rationale to hypothesize that part of the benefit of this pluripotent “age-essential” micronutrient is in decreasing the formation of ceramides in pro-inflammatory states such as aging.
1.2 Dissertation hypothesis and aims

This dissertation has three hypotheses.

First, we hypothesize that **ceramides are accumulating in cardiac mitochondria from aged animals**. This is because mitochondrial dysfunction that results from the addition of ceramide analogs very closely matches the aging phenotype. We examined this hypothesis by addressing the following questions:

1. Do cardiac mitochondria contain ceramide? This question will be answered by analyzing purified cardiac mitochondria from Fischer 344 (F344) rats by LC-tandem mass spectrometry (LC-MS/MS).

2. Is there an age-related ceramide accumulation in cardiac mitochondria? We will use F344 rats as they are a well-documented model of aging maintained by the National Institute on Aging. Ceramides will be quantified by LC-MS/MS from both young (3-6 mo) and old (26-28 mo) rats.

Second, we propose that **depletion of mitochondrial glutathione can activate neutral sphingomyelinase (nSMase), thereby leading to an accumulation of mitochondrial ceramide**. Our lab has previously shown that glutathione levels decline in cardiac mitochondria with age. Interestingly, recent evidence shows that the activity of a major ceramide synthesizing enzyme, nSMase, is inversely regulated by glutathione levels. This hypothesis was examined by addressing the following question:
1. Does a mitochondria-specific glutathione depletion cause “ceramidosis”? To answer this question, mitochondrial GSH will be depleted in F344 rat hepatocytes. Ceramides will be monitored as well as nSMase activity. Hepatocytes were chosen for these experiments due to the large amount of material that can be obtained from a single liver. Cardiomyocytes, while a more appropriate model, are not available in a large enough quantity to make the planned experiments feasible.

Third, we hypothesize that the mitochondrial ceramidosis evident with age can be abrogated by LA treatment. Previous work in our lab, as well as others, shows that lipoic acid treatment restores cellular GSH levels to youthful levels. To address this hypothesis we asked the following questions:

1. Does LA treatment restore mitochondrial GSH to youthful levels? To answer this question we will supplement F344 rats with LA for two weeks followed by cardiac mitochondrial isolation and GSH analysis.

2. Does LA treatment reverse the age-related ceramide accumulation?

3. Does LA restore proper electron transport function?
Chapter 2

Characteristics of the Rat Cardiac Sphingolipid Pool in Two Mitochondrial Subpopulations

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2.1 Abstract

Mitochondrial sphingolipids play a diverse role in normal cardiac function and diseases, yet a precise quantification of cardiac mitochondrial sphingolipids has never been performed. Therefore, rat heart interfibrillar (IFM) and subsarcolemmal (SSM) mitochondria were isolated, lipids extracted, and sphingolipids quantified by LC-tandem mass spectrometry. Results showed that sphingomyelin (~10,000 pmols/ mg protein) was the predominant sphingolipid regardless of mitochondrial subpopulation, and measurable amounts of ceramide (~70 pmols/mg protein) sphingosine, and sphinganine were also found in IFM and SSM. Both mitochondrial populations contained similar quantities of sphingolipids except for ceramide which was much higher in SSM. Analysis of sphingolipid isoforms revealed ten different sphingomyelins and six ceramides that differed from 16 to 24 carbon units in their acyl side-chains. Subfractionation experiments further showed that sphingolipids are a constituent part of the inner mitochondrial membrane. Furthermore, inner membrane ceramide levels were 32% lower versus whole mitochondria (45 pmols/mg protein). Three ceramide isotypes (C_{20^-}, C_{22^-}, and C_{24^-}-ceramide) accounted for the lower amounts. The concentrations of the ceramides present in the inner membranes of SSM and IFM differed greatly. Overall, mitochondrial sphingolipid content reflected levels seen in cardiac tissue, but the specific ceramide distribution distinguished IFM and SSM from each other.
2.2 Introduction

Sphingolipids are a diverse class of lipids that collectively play important roles in membrane ordering reactions, signal transduction, and cell recognition (82, 118). These compounds consist of a sphingosine backbone linked to a fatty acyl side-chain of varying lengths. Subclasses of sphingolipids are further structurally categorized by different head-groups attached to the long-chain base. Metabolically, different sphingolipids often have opposing actions on cell function (119). For instance, ceramide (e.g. \(N\)-acylsphingosine) promotes cell differentiation and growth arrest and is considered as an integral part of apoptosis initiation (120). In contrast, an increased level of sphingosine-1-phosphate tends to induce cellular proliferation and survival (121). Thus, altering levels of a particular sphingolipid subclass relative to another often has significant effects on cell and tissue metabolism (122, 123).

Mitochondria, key organelles involved in cellular bioenergetics and regulation of apoptosis (14), also appear to be important sub-cellular sites for sphingolipid action (124, 125). Both inner and outer mitochondrial membranes contain sphingomyelinases with neutral and acidic pH optima (122, 126), and ceramidases have also been detected in mitochondrial-enriched fractions (87).

In concert with these enzymes, mitochondrial membranes normally contain a variety of sphingolipids. For example, mitochondria from brain, heart, and liver have discernable levels of ceramide (127) and sphingomyelin (128). Additionally, in one of the most complete studies to date, Ardail et al. showed that
rat liver mitochondria contain 3-ketosphinganine, sphinganine, and a variety of ceramide isoforms differing in acyl side-chain length (129). This latter attribute may be important as different side-chains confer specific and sometimes opposing biological actions on sphingolipids (119). Thus, mitochondria appear competent to metabolize at least certain sphingolipids and may therefore be responsive to external stimuli that could affect sphingolipid pools within the organelle.

In vitro studies also show that altering mitochondrial sphingolipid levels markedly affect organelle function. Addition of ceramides composed of certain acyl side-chains (e.g. C_{16}\text{-ceramide}) to mitoplasts results in inhibition of Complex IV (76), and induction of reactive oxygen species (ROS) (74, 76). Furthermore, accumulation of mitochondrial ceramides promotes apoptogenesis by causing dephosphorylation of Bcl-2 family heterodimers (77, 78). Alternatively, sphingosine-1-phosphate protects cells from mitochondrial-driven apoptosis (130). These examples highlight the concept that altering the mitochondrial sphingolipid pool significantly affects both cellular bioenergetics and the propensity for programmed cell death. However, even though it appears that certain sphingolipids are constituents of mitochondrial membranes and can affect their function, a precise analysis of the overall mitochondrial sphingolipid pool has not been fully achieved.

The current study was undertaken to provide a more thorough characterization of the mitochondrial sphingolipid pool using LC-tandem mass spectrometry (LC-MS/MS). Cardiac mitochondria were chosen for this analysis because the heart contains two unique mitochondrial subpopulations that are
either located along the myofibrils (interfibrillar [IFM]) or adjacent to the sarcoplasmic reticulum (subsarcolemmal [SSM]) (27). Differences with respect to respiratory activity, propensity for oxidative damage, and their contribution to pathophysiologies have been reported for these two mitochondrial subpopulations (18, 30). As sphingolipids may theoretically be involved in the divergent functional properties of the IFM and SSM, the current study was intended to not only understand cardiac mitochondrial sphingolipids *per se*, but also to discern potential differences in sphingolipid content and composition in IFM and SSM.
2.3 Materials and Methods

Reagents

The following reagents were used: genistein, Triton X-100, and Tween 20 (Sigma-Aldrich, St. Louis, MO); subtilisin A (type VIII) and bovine serum albumin (EMD Biosciences, La Jolla, CA); and digitonin (Thermo Fisher Scientific, Pittsburg, PA). Purified sphingolipid standards were purchased from Avanti Polar Lipids (Alabaster, AL). Rabbit polyclonal antibody to the voltage-dependent anion channel protein (VDAC) and mouse monoclonal antibody to protein disulfide isomerase (PDI) were purchased from Abcam, Inc (Cambridge, MA).

Ethical treatment of vertebrate animals

Fischer 344 rats (male, 4-6 months old) were obtained from the National Institute on Aging animal colonies. Animals were housed in approved facilities and maintained by the Oregon State University Laboratory Animal Resources Center. All animal procedures were performed in accordance with OSU guidelines for animal experimentation and approved by the institutional animal care and use committee (IACUC).
Mitochondrial and microsome isolation

Cardiac mitochondria were isolated using differential centrifugation and a brief protease digestion as previously described (27). One noted change from this method is the use of subtilisin A instead of nagarse to release mitochondria from the myofibrils. Microsomes were obtained by subjecting the supernatant from the SSM isolation to centrifugation at 100,000 x g for one hour. All steps of the isolation were performed on ice or at 4°C. Protein values were determined using the BCA protein assay kit.

Mitochondrial inner membrane isolation

The outer mitochondrial membrane was selectively removed using digitonin (131). Six mg/ml digitoninin at 37°C in isotonic buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM tris-HCl, 5 mM KH$_2$PO$_4$, pH 7.2) was determined to be optimal for removing outer membranes (Figure 2.1C). This procedure resulted in a highly purified inner mitochondrial membrane (IMM) fraction with less than 2% contamination of the outer membrane. The IMM could not be further purified by Percoll density centrifugation as yields were too low to allow LC-MS/MS analysis.
**Assessment of mitochondrial purity**

Sphingolipids are found in all cell membranes; therefore, it was necessary to determine the extent of non-mitochondrial membrane contamination in IFM and SSM. The levels of microsomes and lysosomes in the mitochondrial preparations were chosen for the assessment because they are the most likely sources of contamination for any cardiac mitochondrial isolation scheme (82). Results showed that the extent of ER contamination was minimal as shown by the lack of PDI, a marker protein for the ER (Figure 2.1A). Similarly, mitochondrial contamination with lysosomes, as measured by N-acetyl-β-D-glucosaminidase (NAG) activity, was limited to ≤ 1.5% of that found in tissue homogenates (Figure 2.1B). These results, along with rigorous purification of the IMM from isolated mitochondrial subpopulations (Figure 2.1C), indicate that cardiac mitochondria and their isolated inner membranes were sufficiently free of extramitochondrial contaminants to properly assess sphingolipid content. However, outer mitochondrial membranes were found to be of insufficient purity to properly monitor sphingolipids. Thus, only the sphingolipid profile from IMM or intact mitochondria could be reported.

**Western blotting**

Western blotting was performed as detailed in Petersen Shay and Hagen (132).
Phospholipid phosphate determination

The amount of phospholipid phosphate was determined using a colorimetric assay developed by Bartlett et al. (133).

Lipid extraction

All samples were prepared as in Merrill et al. (134) with the exception that samples were not digested in KOH. Briefly, a sphingolipid internal standard (25 μl) was added to mitochondria (1 mg protein). Lipids were extracted with 1.5 ml of chloroform:methanol (1:2). After an overnight incubation at 48°C, phases were separated by adding chloroform (2 ml) and H₂O (4 ml). The chloroform layer was aspirated and dried under N₂. The sample was reconstituted in 200 μl chloroform:methanol (3:1) and diluted 1:4 with acetonitrile:methanol:acetic acid (97:2:1) containing 5 mM ammonium acetate. Recovery of lipids was monitored by LC-MS/MS (see below); all sphingolipids of the internal standard were detected in quantifiable and reproducible amounts.

LC-Tandem mass spectrometry

Lipids were separated using a Shimadzu HPLC and a Supelco Discovery column (2 mm x 50 mm). The flow rate was set at 300 μl per minute. Mobile phase (A) contained methanol:water (60:40) while mobile phase (B) was composed of methanol:chloroform (60:40). Both solvents contained 0.2% (v/v)
formic acid and 10 mM ammonium acetate. The column was pre-equilibrated at 100% (A) followed by a sample injection. The solvent was maintained at 100% (A) for one minute, followed by a linear gradient to 40% (B) after 8.0 minutes. Finally, the solvent mix was brought to 70% (B) after 13 minutes and then maintained at this percentage for the remainder of the 20 minute run.

The HPLC system was coupled through a TurboIon Spray source to a triple-quadrupole mass spectrometer operated in positive mode (Applied Biosystems/MDS Sciex, API 3000). Analytes were detected using Multiple Reaction Monitoring (MRM), which selectively detects fragment ions from the collision-induced dissociation (CID) of the parent molecular ion. Please see Supplemental Table 1 (appendix) for a list of molecular ions and CID transitions. Quantification was based on comparison to sphingolipid standards.

**Statistics**

Data are presented as mean ± SEM. Samples were assessed for statistical significance using Student’s t test. A p value <0.05 was considered statistically significant.
2.4 Results and Discussion

Analysis of rat heart sphingolipids

IFM and SSM sphingolipid profiles

Mitochondrial sphingolipids were monitored using LC-MS/MS and quantified according to Leibisch et al. (135). Sphingomyelin was the predominant sphingolipid (10,000 pmol/mg protein) in cardiac IFM and SSM (Figure 2.2A). While ceramide (Figure 2.2B), sphingosine (Figure 2.2C), and sphinganine (Figure 2.2D) were measurable, these sphingolipids were at least 150-fold lower than sphingomyelin levels. Cardiac IFM and SSM thus contain similar quantities of each sphingolipid sub-class, which suggests that differences in their general sphingolipid profiles are not likely responsible for the functional divergence between these two mitochondrial subpopulations. In addition to these aforementioned sphingolipids, attempts were made to quantify sphingosine-1-phosphate, sphinganine-1-phosphate, as well as glucosylceramides in both IFM and SSM. However, while detectable in limited quantities, all fell below the quantitative limit of our assay. Thus, SSM and IFM primarily contain sphingomyelin and ceramide with minor amounts of other sphingolipids.

Comparison of mitochondrial sphingolipids to other cardiac membranes

In order to place the mitochondrial sphingolipid profile in context to other cardiac membranes, sphingolipids were measured in lipid extracts from heart
Figure 2.1 Intact mitochondria and inner membrane preparations are sufficiently pure for sphingolipid determination. Microsomal and lysosomal contamination of the IFM was monitored by measuring protein disulfide isomerase (PDI) and N-acetyl-β-D-glucosaminidase (NAG) activity, respectively. (A) Western blot analysis shows no detectable PDI in IFM preparations. n=3; (B) The percentage of NAG in IFM and SSM were ≤1.5% of that found in cardiac tissue homogenates. N=3; (C) Optimal digitonin levels to solubilize mitochondrial outer membranes from the IMM were determined. Western blot analysis showed successively greater removal of VDAC, a protein marker of mitochondrial outer membranes, with increasing digitonin levels added; however, COX IV (an inner membrane marker) remained constant regardless of digitonin levels. The blot shown is typical of four preparations.
Figure 2.2 Sphingolipid profiles in mitochondrial and extra-mitochondrial cardiac membranes. Lipids extracted from IFM, SSM, or their respective inner membranes were subjected to LC-MS/MS and sphingolipids quantified relative to standards. Sphingolipids were also monitored in cardiac microsomes and lipid extracts from tissue homogenates. (A) Sphingomyelin; (B) ceramide; (C) sphingosine; and (D) sphinganine are shown. Data are Mean ± SEM, n=6; an asterisk (*) denotes significant differences between intact mitochondria and IMM; a superscript (a) indicates a statistically significant difference from whole tissue, p < 0.05.
tissue and isolated microsomes. Figure 2.2 shows that mitochondrial sphingolipid content generally reflects the sphingolipid profile in cardiac tissue, but is noticeably lower relative to the levels seen in microsomes. Thus, even though mitochondria often form contiguous networks with the ER (136), the relatively low levels of mitochondrial sphingolipids suggest that lateral diffusion from the ER is not the sole means of regulating mitochondrial sphingolipid levels.

**Sphingolipids of the inner mitochondrial membrane**

Because the IMM is relatively impervious to proton and solute translocation, we hypothesized that sphingolipids, which participate in membrane ordering, may be components of this specialized membrane (137). Determination of sphingolipid subclasses revealed that inner membranes of both IFM and SSM contained sphingomyelin, ceramide, sphingosine and sphinganine (Figure 2.2). While this profile was similar to intact mitochondria, IMM were markedly enriched in sphingomyelin versus whole mitochondria. This relative enrichment was observed for both SSM and IFM inner membranes, which were 58% and 64% higher, respectively.

Large differences in ceramide levels between IFM and SSM inner membranes were observed (Figure 2.2B). SSM inner membrane ceramides were elevated at least 3-fold relative to the IFM (Figure 2.2B), suggesting potential ceramide-specific characteristics in the SSM. While not currently examined, it is known that elevated ceramides inhibit the respiratory chain (76), which indicates
that higher SSM ceramide levels may be a factor in the lower rates of respiratory activity evident for this subpopulation (27). Further work will be necessary to define the potential functional consequences of the asymmetric distribution of ceramides in IFM and SSM inner membranes.

**Quantification of mitochondrial sphingolipid isoforms**

A significant advantage of using LC-MS/MS is the opportunity to monitor not only a particular sphingolipid class, but also the distribution of sphingolipid isotypes based on acyl side-chain length. Analysis of specific sphingolipid species revealed that rat cardiac mitochondria contain numerous isoforms differing in side-chain unsaturation and acyl chain length (16-24 carbons).

**Specific sphingomyelin isoforms in cardiac mitochondria**

As shown in Table 3.1, no discernable differences in sphingomyelins were evident between the mitochondrial subpopulations. $C_{20}$- and $C_{22}$-sphingomyelin were the predominant mitochondrial isotypes, each of which comprised approximately 33% of the total sphingomyelin pool. As with the general sphingomyelin profile (Figure 2.2), the distribution patterns of specific sphingomyelin isoforms generally reflect that seen for cardiac tissue, and not for microsomes (Table 3.1). Purified cardiac mitochondria contain low amounts of $C_{16}$-, $C_{18}$-, and $C_{24}$-sphingomyelin when compared to microsomes. The limited
levels of these sphingomyelins are notable because ceramides with similar acyl side-chains accumulate during stress insult (81, 138). Thus stress-activated sphingomyelinase production of ceramide may not be the sole route of ceramide elevation in mitochondria, which could implicate extra-mitochondrial transfer (86).

*Specific ceramide isoforms in cardiac mitochondria*

Distinct differences in ceramide isoform patterns were evident when compared to the mitochondrial sphingomyelin profile (Figure 2.3). Regardless of subpopulation, mitochondria contain six ceramides having acyl chain lengths that range from 16- to 24-carbon units (Figure 2.3 and Table 2.1). As opposed to the sphingomyelins which had five unsaturated species, the ceramide pool contained only one isoform (C$_{24:1}$-ceramide) with a site of unsaturation (Figure 2.3). The general profile of mitochondrial ceramide is similar to that found in brain (139) and liver (129); however, the absolute amounts of a particular ceramide isotype are different between cardiac mitochondria and mitochondria from other organs (129, 139).

In terms of ceramide distribution, C$_{24}$-ceramide is the most abundant mitochondrial ceramide (~40%) in both IFM and SMM, while C$_{16}$- and C$_{18}$-ceramide are the most limited (Figure 2.3A). IFM and SSM contain similar amounts of C$_{16}$- and C$_{18}$-ceramide but all other species are higher in the IFM as compared to the SSM (>50%, p < 0.05).
Table 2.1 Sphingomyelin Content of various cardiac myocyte membranes.

<table>
<thead>
<tr>
<th>Sphingomyelin Species</th>
<th>IFM</th>
<th>IFM-IMM</th>
<th>SSM</th>
<th>SSM-IMM</th>
<th>Tissue</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{16} )-sphingomyelin</td>
<td>25 ± 4</td>
<td>907 ± 62</td>
<td>45 ± 12</td>
<td>1749 ± 400</td>
<td>1370 ± 88</td>
<td>11002 ± 872</td>
</tr>
<tr>
<td>( C_{18} )-sphingomyelin</td>
<td>51 ± 4</td>
<td>722 ± 70</td>
<td>64 ± 11</td>
<td>1039 ± 229</td>
<td>822 ± 76</td>
<td>6504 ± 381</td>
</tr>
<tr>
<td>( C_{20} )-sphingomyelin</td>
<td>3394 ± 216</td>
<td>4496 ± 670</td>
<td>3418 ± 281</td>
<td>4160 ± 761</td>
<td>7537 ± 2422</td>
<td>15530 ± 2153</td>
</tr>
<tr>
<td>( C_{22} )-sphingomyelin</td>
<td>3465 ± 203</td>
<td>4733 ± 594</td>
<td>3591 ± 315</td>
<td>4223 ± 680</td>
<td>8497 ± 2642</td>
<td>18514 ± 2523</td>
</tr>
<tr>
<td>( C_{24} )-sphingomyelin</td>
<td>161 ± 11</td>
<td>956 ± 90</td>
<td>124 ± 19</td>
<td>1035 ± 186</td>
<td>1155 ± 139</td>
<td>7476 ± 574</td>
</tr>
<tr>
<td>( C_{16:1} )-sphingomyelin</td>
<td>0.95 ± 0.04</td>
<td>17 ± 1.2</td>
<td>1.28 ± 0.22</td>
<td>25 ± 6</td>
<td>24 ± 1.3</td>
<td>192 ± 11</td>
</tr>
<tr>
<td>( C_{18:1} )-sphingomyelin</td>
<td>2.81 ± 0.31</td>
<td>81 ± 8.5</td>
<td>3.51 ± 0.59</td>
<td>76 ± 17</td>
<td>81 ± 6.5</td>
<td>695 ± 53</td>
</tr>
<tr>
<td>( C_{20:1} )-sphingomyelin</td>
<td>278 ± 71</td>
<td>457 ± 97</td>
<td>258 ± 67</td>
<td>341 ± 89</td>
<td>837 ± 336</td>
<td>1630 ± 270</td>
</tr>
<tr>
<td>( C_{22:1} )-sphingomyelin</td>
<td>1746 ± 142</td>
<td>2545 ± 394</td>
<td>1901 ± 128</td>
<td>2186 ± 429</td>
<td>3514 ± 1245</td>
<td>5923 ± 941</td>
</tr>
<tr>
<td>( C_{24:1} )-sphingomyelin</td>
<td>1020 ± 53</td>
<td>1695 ± 178</td>
<td>1158 ± 81</td>
<td>1861 ± 284</td>
<td>2368 ± 853</td>
<td>6395 ± 900</td>
</tr>
</tbody>
</table>

* All data are represented as mean ± SEM, n = 6.
Ceramide isoforms in IFM and SSM inner membranes

Analysis of inner membrane ceramides showed that both IFM and SSM contained all 6 isotypes that were present in intact mitochondria. However, overall, inner membrane ceramide levels and the amount of a particular ceramide varied markedly between IFM and SSM. As shown in Figure 2.2B, the SSM inner membrane contained significantly higher ceramide levels than intact mitochondria. This enrichment stemmed from elevated levels of all six ceramide species (Figure 2.3B). In particular, C₁₆- and C₁₈-ceramide were 21- and 9-fold higher, respectively, in the inner membrane than corresponding levels in the intact SSM. Also, all other ceramide isoforms were at least 2-fold higher than whole SSM.

When comparing ceramides in intact mitochondria with the inner membrane fraction, it was found that the IFM inner membrane is enriched in C₁₆- and C₁₈-ceramide, while being limited in ceramides with longer acyl chain lengths (Figure 2.3C). This apparent ceramide isoform asymmetry in mitochondrial membranes was independent of protein content, as a similar distribution pattern was also observed when based on phospholipid phosphate levels in lieu of protein amounts (Supplemental Figure 1 in the appendix).
Figure 2.3 Ceramide isotypes in mitochondrial and extra-mitochondrial cardiac membranes. Ceramides were quantified in indicated cardiac membranes using LC-MS/MS. Results showed that all membranes contain six ceramide species with side chains from 16 to 24 carbons in length. (A) Ceramide distribution in SSM and IFM. (B) Profile of SSM and IFM ceramide isotypes in mitochondrial inner membranes. (C) Ratio of inner membrane ceramides to those of intact mitochondria. (D) Ceramide levels found in extra-mitochondrial membranes. Data represents the mean ± SEM, n = 6; an asterisk (*) denotes a significant difference to the corresponding isotype in IFM, p < 0.05. Bars represent: C_{16}-ceramide (black), C_{18}-ceramide (vertical grey lines), C_{20}-ceramide (checkered), C_{22}-ceramide (grey), C_{24}-ceramide (angled lines), C_{24:1}-ceramide (clear).
IFM inner membrane ceramide isotypes were generally less abundant versus the intact organelle (Figure 2.3D). For example, C_{16}-ceramide was approximately 50% of the total; only C_{18}-ceramide values in the inner membrane approached that of intact IFM (Figure 2.3D). The tissue as a whole shared a similar profile to that of both the SSM and IFM, and hence was distinct from the inner membrane patterns. The microsomal ceramide pool contained the same acyl side-chains as the mitochondria and tissue but all isotypes were found at relatively high levels. Also, the amounts of each ceramide isotype relative to one another were different than those found in tissue and intact mitochondria (Figure 2.3D). These results, once again, indicate that microsomal sphingolipids are a distinct pool from the mitochondria.
2.5 Conclusions

Given the critical role that these lipids play in mitochondrial function, it is important to understand the general composition and membrane distribution of cardiac mitochondrial sphingolipids. The present work shows that cardiac mitochondria contain an array of sphingolipids, which reflects the distribution seen in heart and other tissues (129, 139). Despite this resemblance, the concentration of a given mitochondrial sphingolipid isotype varies widely and depends on the tissue studied. For example, liver mitochondria predominantly contain C_{16}-, C_{18}-, C_{18:1}-ceramide with C_{24}-ceramide being a minor constituent; C_{22}-ceramide is not detected (129). In contrast, we report that C_{24}- and C_{22}-ceramide are the most abundant ceramides found in cardiac mitochondria. The reason(s) for these differences are not presently known, but may be due to tissue specific expression of ceramide synthases (140), which would significantly influence composition of mitochondrial ceramide isotypes in a particular organ.

The functional consequences for different ceramides in mitochondria have yet to be explored. However, the relative abundance of a ceramide species may modulate the sensitivity of mitochondria to apoptotic stimuli (119) or affect ROS production. Thus, a post-mitotic organ, such as the heart, may limit shorter-chain ceramides to modulate pro-apoptotic signals versus a mitotically active organ like the liver, which can readily undergo tissue regeneration.

Sphingolipids play key roles in membrane curvature, regulation of energy production, and membrane permeability. Thus, alterations in basal sphingolipid
levels may be the root-cause for the metabolic and structural differences found between SSM and IFM. Our results show that the sphingomyelin, sphingosine, and sphinganine content of cardiac mitochondria are nearly identical, and therefore cannot contribute to the functional differences of these two subpopulations. However, IFM and SSM display distinct ceramide profiles, which could contribute to differences in respiratory characteristics and sensitivity to pro-apoptotic stimuli. This in turn, may play a vital role in disease states, such as the decline in cardiac function seen in aging, where IFM becomes specifically dysfunctional.

We are currently attempting to determine both the mechanism(s) causing IFM and SSM ceramide asymmetry, and the functional consequences of such an asymmetry. With regards to the mechanism, the recent work by Wu et al. may be instructive, as this group showed that a novel isoform of neutral sphingomyelinase exists in mitochondria (I41). This presents the intriguing possibility of a differential expression or regulation of neutral sphingomyelinase in IFM and SSM. Thus, ceramide pool sizes and specific isoforms could be regulated via the catabolism of sphingomyelin to ceramide.
Age-related Ceramide Accumulation in the Inner Mitochondrial Membrane May Contribute to Cardiac Mitochondrial Decay

Jeffrey S. Monette, Luis A. Gómez, Régis F. Moreau, Kevin C. Dunn, Judy A. Butler, Liam A. Finlay, Alexander J. Michels, Kate Petersen Shay, Eric J. Smith, and Tory M. Hagen

Data from this chapter are excerpts from the published article entitled *(R)-α-Lipoic Acid Treatment Restores Ceramide Balance in Aging Rat Cardiac Mitochondria.*


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3.1 Abstract

Ceramide added to mitochondria initiates a phenotype [e.g. electron transport chain (ETC) inhibition and superoxide production] similar to that seen in aging. We hypothesized that age-related mitochondrial decay was thus partly due to aberrant mitochondrial ceramide metabolism. To test this hypothesis we developed an LC-MS/MS assay to quantify cardiac mitochondrial ceramides from young (3 mo) and old (24-26 mo) F344 rats. Results show that mitochondria contain 6 ceramide species with acyl groups from 16 to 24 carbons in length. The two ceramides known to inhibit the ETC and induce apoptosis, $C_{16}$-ceramide and $C_{18}$-ceramide, are found solely in the inner mitochondrial membrane (IMM). Interestingly, only $C_{16}$-ceramide and $C_{18}$-ceramide increased with age and were 72.3% (p < 0.05, n=4) and 73.4% (p < 0.05, n=4) higher, respectively, than in young controls. Elevating IMM ceramide in mitochondria from young rat hearts by incubating membranes with exogenous sphingomyelinase significantly inhibited Complex IV activity by 50% (P ≤ 0.01; N=3). This inhibition was similar to the aging phenotype. Thus, age–related IMM ceramide accumulation may be a novel initiator of ETC inhibition, leading to known characteristics of mitochondrial decay evident in the aging heart.
3.2 Introduction

Mitochondria from aged tissue undergo a systematic decline in overall function, which manifests in the heart as an increased rate of reactive oxygen species (ROS) formation, concomitant oxidative damage, and impaired electron transport (10, 16-22). All of these factors limit the ability of mitochondria to meet cellular energy needs. Interestingly, these mitochondrial traits of aging are also evident, albeit more severely, in inflammatory pathologies (54-56). As it is recognized that the aging heart is subjected to a low-grade chronic inflammation, it is reasonable to argue that inflammatory bio-factors may be involved in both the initiation and progression of mitochondrial decay. One such bio-factor that appears to be a hallmark of pro-inflammatory conditions is ceramide, a pro-apoptotic and growth arrest sphingolipid (42-45, 47, 48, 50, 82, 142), which increases ROS formation, oxidative stress, and altered energy metabolism upon its accumulation in membranes (74, 76).

Generally, acute inflammatory stimuli generate ceramide at the plasma membrane or endoplasmic reticulum by sphingomyelin hydrolysis or de novo synthesis, respectively (37, 40, 59, 101, 143-145). Recent evidence shows that mitochondria may also be an important site of sphingolipid action (76, 124, 146-148). Our laboratory recently showed that cardiac mitochondria normally contain a variety of sphingolipids, including sphingomyelin and ceramide (149). Mitochondria from other organs also contain ceramide as well as neutral sphingomyelinase (nSMase), which hydrolyzes sphingomyelin to ceramide (87, 129, 150). This suggests that mitochondria have the means to alter ceramide
levels in response to pro-inflammatory stimuli. Moreover, *in vitro* experiments suggest that even small elevations of mitochondrial ceramide is able to adversely affect electron transport chain (ETC) activity, heighten ROS appearance, and also initiate mitochondrial-mediated apoptosis (74, 76, 124, 147, 148). Thus, age-associated inflammation of the heart and mitochondrial decay may be connected via ceramidosis (i.e., the accumulation of ceramide). If so, this would provide a novel target for therapies to improve cardiac mitochondrial function and bioenergetics, which otherwise decline with age.

Despite this potential association, the role that ceramide plays in age-related mitochondrial decay has not been studied. Because many of the phenotypes of mitochondrial dysfunction can be plausibly linked to ceramidosis, the goal of the current study was to determine ceramide levels in interfibrillary mitochondria isolated from young and old rat hearts. Moreover, as mitochondria are double-membraned organelles, we further pursued the hypothesis that ceramide accumulation would be evident in the inner mitochondrial membrane (IMM) and adversely affect ETC activity.
3.3 Materials and Methods

Chemicals and antibodies

Digitonin, genistein, Subtilisin A (type VIII), Triton X-100, and Tween 20 were from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (fraction V, fatty acid free) was obtained from EMD Biosciences (La Jolla, CA). Purified ceramide standards were purchased from Avanti Polar Lipids (Alabaster, AL). NBD-sphingomyelin and NBD-ceramide were purchased from Life Technologies (Carlsbad, CA). Rabbit polyclonal antibody to the voltage-dependent anion channel protein (VDAC) and mouse monoclonal antibody to protein disulfide isomerase (PDI) were purchased from Abcam, Inc. (Cambridge, MA). All other compounds were reagent grade or of the highest purity obtainable.

Ethical treatment of vertebrate animals

Young (4-6 mo) and old (26-28 mo) Fischer 344 male rats were obtained from the National Institute on Aging animal colonies. Animals were housed in approved facilities and maintained by the Oregon State University Laboratory Animal Resources Center. All animal procedures were performed in accordance with OSU guidelines for animal experimentation and approved by the institutional animal care and use committee (IACUC).
Mitochondrial isolation

Cardiac mitochondria were isolated using differential centrifugation as described by Palmer et al. (27) with modifications as outlined in Monette et al. (149). This procedure resulted in an enriched interfibrillar mitochondrial fraction. All steps of the isolation were performed on ice or at 4°C. Protein values were determined using the BCA protein assay kit (Thermo Scientific; Rockford, IL).

Inner mitochondrial membrane (IMM) isolation

The outer mitochondrial membrane (OMM) was selectively removed using digitonin (131). Six mg/ml digitonin at 37°C in isotonic buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM tris-HCl, 5 mM KH$_2$PO$_4$, pH 7.2) was optimal for removing the OMM. This procedure resulted in a highly purified IMM fraction with less than 2% contamination from OMM as determined by immunoblotting for VDAC. The IMM could not be further purified by Percoll density centrifugation as yields were too low to allow for LC-MS/MS analysis.
Mitochondrial Complex IV activity

Cytochrome c oxidase (Complex IV) activity was measured using a commercially available kit from Sigma-Aldrich that follows the oxidation of cytochrome c.

Activity assay for neutral sphingomyelinase and ceramidase

Fluorescently-labeled sphingomyelin and ceramide (NBD-sphingomyelin and NBD-ceramide, respectively) were used as substrates to determine the activities of ceramide metabolizing enzymes by the method of Lightle et al. (151).

Measurement of glutathione (GSH)

GSH was conjugated to dansyl chloride and measured by using HPLC and fluorescence detection as described by Dixon et al. (152).

Lipid extraction

All samples were prepared as in Merrill et al. (134) except that samples were not saponified in KOH.
**LC-tandem mass spectrometry**

Lipids were separated by HPLC using a Supelco Discovery column (2 mm x 50 mm; Sigma-Aldrich). The flow rate was set at 300 μl per minute. Mobile phase A contained methanol:water (60:40) while mobile phase B was composed of methanol:chloroform (60:40). Both solvents contained 0.2% (v/v) formic acid and 10 mM ammonium acetate. The elution gradient was as follows: the column was pre-equilibrated at 100% mobile phase A followed by sample injection (5 μl); 100% mobile phase A was maintained for one minute, followed by a linear increase to 40% mobile phase B over a 7 minute period; followed by a linear increase to 70% mobile phase B over the next 6 minutes; 70% mobile phase B was maintained for the remainder (6 minutes) of the 20 minute run.

Analytes were detected on a triple-quadrupole mass spectrometer operated in positive mode (Applied Biosystems/MDS Sciex, API 3000) using multiple reaction monitoring, which selectively detects fragment ions from the collision-induced dissociation of the parent molecular ion. For a list of molecular ion transitions, please see Monette et al. (149). Quantitation was based on comparison to synthetic sphingolipid standards.

**Statistics**

Data are presented as means ± SEM. Samples were assessed for statistical significance using a one-way ANOVA test. Multiple comparisons were made
using a Tukey’s post hoc test or the Student’s t-test. A p value $\leq 0.05$ was considered statistically significant.
3.4 Results

Profile of cardiac mitochondrial sphingolipids

In keeping with results from our previous work (149), both intact cardiac mitochondria as well as purified IMM contained six ceramide isotypes with N-acyl-chain lengths varying from 16- to 24-carbon units (Figure 3.1). The ceramides were predominantly saturated, with only one species, C_{24:1}-ceramide, containing an unsaturated N-acyl side-chain. C_{24}-ceramide was the predominant isoform found in cardiac mitochondria and comprised 38% of the total ceramide pool. Quantifying ceramides in the IMM showed that C_{16}-, C_{18}-, and C_{24:1}-ceramide were in nearly equal concentrations as in whole mitochondria. However, the IMM contained only 35 to 50% of C_{20}-, C_{22}-, and C_{24}-ceramide versus intact mitochondria, suggesting that these particular ceramides are enriched in the OMM fraction. However, further attempts to measure relative levels of ceramide isotypes in the OMM were not successful because of extra-mitochondrial membrane contamination. Thus, cardiac mitochondria contain a variety of ceramide isoforms, and it appears that IMM ceramides are particularly enriched in C_{16}-, C_{18}-, and C_{24:1}-ceramide.

Age-related mitochondrial ceramide accumulation

On an age basis, total mitochondrial ceramide increased by 32% (Figure 3.2). Analysis of individual isoforms showed that the age-related elevation in ceramide stemmed from increases in all ceramide species (Table 3.1). For
Figure 3.1 Asymmetric distribution of cardiac mitochondrial ceramides.

Ceramide content of purified intact mitochondria and inner mitochondrial membrane (IMM) as quantified by LC–MS/MS. Both fractions contain six ceramide isoforms ranging from 16- to 24-carbon units in length, with C$_{24}$-ceramide being the predominant species in intact mitochondria. C$_{16}$-, C$_{18}$-, and C$_{24:1}$-ceramide are found in near-equivalent quantities in the IMM as compared to intact mitochondria, whereas C$_{20}$-, C$_{22}$, and C$_{24}$-ceramide are present in much lower quantities. This suggests that these latter ceramides are primarily found in the outer mitochondrial membrane. Data represent the means ± SEM, n = 4.
Figure 3.2 Total ceramide increases in mitochondria from aged animals.

Young and old rats were sacrificed, mitochondria were isolated, and lipids were extracted and analyzed by LC-MS/MS. Results show an increase of ceramides by 32% in old animals. Data represent the means ± SEM, n = 4; an asterisk (*) denotes a significant difference by Student’s t-test, p < 0.03.
Table 3.1 Cardiac mitochondrial ceramide levels

<table>
<thead>
<tr>
<th>Ceramide Species</th>
<th>Young Control</th>
<th>Old Control</th>
<th>Δ% from Young Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{16}-ceramide</td>
<td>7.4 ± 0.6</td>
<td>12.7 ± 1.9*</td>
<td>72.3</td>
</tr>
<tr>
<td>C_{18}-ceramide</td>
<td>7.8 ± 1.3</td>
<td>13.5 ± 1.2*</td>
<td>73.4</td>
</tr>
<tr>
<td>C_{20}-ceramide</td>
<td>12.5 ± 1.5</td>
<td>14.8 ± 1.1</td>
<td>18.1</td>
</tr>
<tr>
<td>C_{22}-ceramide</td>
<td>27.7 ± 2.6</td>
<td>30.0 ± 0.9</td>
<td>8.0</td>
</tr>
<tr>
<td>C_{24}-ceramide</td>
<td>40.6 ± 6.4</td>
<td>48.8 ± 5.6</td>
<td>20.0</td>
</tr>
<tr>
<td>C_{24:1}-ceramide</td>
<td>10.9 ± 1.8</td>
<td>19.4 ± 4.2</td>
<td>77.7</td>
</tr>
</tbody>
</table>

All data are represented as means ± SEM, n = 4.

*p < 0.05 vs. young control
analysis of significance of ceramide levels, the Student’s t-test was employed in place of a one-way ANOVA test because of small sample size and unequal variances in the LA groups versus non-treated animals. The largest elevations were those of C_{16}-, C_{18}-, and C_{24:1}-ceramide, which increased by ≥ 70% when compared to young controls. Despite the relatively large apparent accumulation in C_{24:1}-ceramide, statistical significance was not reached because of high variability. There was a trend for C_{20}-, C_{22}-, and C_{24}-ceramide to increase modestly (20% or less), but once again changes in these particular ceramide isoforms did not reach statistical significance (Table 3.1). It is interesting to note that the ceramide species that are found in greater abundance in the IMM (C_{16}-, C_{18}-, and C_{24:1}-ceramide, see Figure 3.1) are the ones that increased the most with age. This suggests that the age-related ceramide accumulation primarily occurs in the IMM.

Mitochondrial ceramide accumulation in vitro leads to electron transport inhibition

Because the age-related ceramide accumulation appears to occur in the IMM and previous reports show that short chain ceramides (e.g. C_{2}- and C_{6}-ceramide) inhibit ETC activity (74, 76), we hypothesized that there was an association between the age-dependent decline in ETC activity and ceramide accumulation. Using Complex IV activity as a surrogate for overall flux of electrons through the ETC, we found its activity declined by 28% (p < 0.05) on an
age basis (Figure 3.3), which is in keeping with previous literature reports (18, 30). As this result does not prove that ceramide accumulation is responsible for ETC inhibition, IMM were incubated with bacterial sphingomyelinase (bSMase) to acutely elevate ceramide levels so that a cause-and-effect relationship between ETC inhibition and ceramide might be discerned. Incubation of bSMase with mitochondria caused ceramide levels to increase by 13.8-fold versus controls (Figure 3.4A). Elevation of ceramide levels via bSMase resulted in a marked 62% loss in Complex IV activity versus controls (Figure 3.4B). Thus, increases in IMM ceramides correlate with a decline in ETC function, thereby suggesting that the age-related accumulation of IMM ceramides may be linked to the loss of Complex IV activity evident in aging heart mitochondria.
Fig. 3.3 Complex IV activity declines in cardiac mitochondria from aged animals. Complex IV activity was monitored in mitochondria isolated from young and old rats by following the oxidation of cytochrome c. Results show a 28% decline in enzymatic activity with age. Data represent the means ± SEM, \( n = 4 \); an asterisk (*) denotes a significant difference from young controls, \( p \leq 0.05 \).
Figure 3.4 Acute ceramide accumulation leads to an inhibition in Complex IV activity. Isolated mitochondria were incubated with a bacterially-derived sphingomyelinase, bSMase, followed by lipid extraction and ceramide quantification by LC-MS/MS. (A) Total ceramide levels were markedly increased by a 20-min bSMase incubation. (B) A time-course of Complex IV activity following incubation with bSMase. Results show a rapid decline in Complex IV activity. Data represent the means ± SEM, n = 3.
3.5 Discussion

To our knowledge, this is the first study showing that cardiac mitochondrial ceramides increase with age. Even though the accumulation was seemingly modest, nevertheless, it may be sufficient to adversely affect mitochondrial function. Tissue analysis following ischemia/reperfusion injury (153), myocardial infarct (154, 155), Type II diabetes (156), as well as in vitro experiments where isolated mitochondria were treated with ceramide-laden liposomes (74, 76), reinforce the view that perturbing normal mitochondrial sphingolipid status, even slightly above the norm, significantly alters mitochondrial function. For example, Yi et al. showed that when rat mesangial cells were incubated with homocysteine, a common marker for cardiovascular disease, there was a 47% increase in ceramides, which also increased ROS formation (57). Also, Straczkowski et al. reported that men at risk for diabetes have a 50 to 200% increase in type II muscle ceramide levels (45). Even though the age-related mitochondrial ceramide accumulation reported here is not as elevated as that found in acute pathologies, this ceramidosis may be sufficient to adversely affect mitochondrial function. Thus, we contend that increased mitochondrial ceramide should be recognized as one of the underlying factors leading to mitochondrial dysfunction with age.

A key result of the present study was the discovery of the asymmetric nature of the evident ceramidosis. C_{16}-, C_{18}-, and C_{24:1}-ceramide were elevated the greatest with age (Table 3.1); moreover, most of this accumulation occurred in the IMM. These intriguing results may be highly significant as there is growing
evidence suggesting that both the acyl chain length and the degree of its unsaturation are important for specific ceramide action in cells. For example, Senkal et al. showed that squamous cell carcinomas were specifically killed by an increase in C\textsubscript{18}-ceramide, but not by orthologs containing even one site of unsaturation (81). C\textsubscript{16}- and C\textsubscript{18}-ceramide appear to be pro-apoptogenic ceramides and commonly increase during pathological conditions (81, 157, 158). In this regard, our current evidence supports the concept that specific ceramide species may be important for apoptotic signaling and inflammation. Indeed, the finding that three isoforms are primarily responsible for mitochondrial ceramidosis is significant as these isoforms could not only promote decline in ETC function as we have observed, but also may play a role in increasing ROS and/or promoting apoptosis, and resulting myocyte loss. We are currently conducting research aimed at understanding the roles played by individual ceramide species in mitochondria.
Chapter 4

Depletion of Mitochondrial Glutathione Leads to Ceramide Formation via Neutral Sphingomyelinase Activation

Jeffrey S. Monette, Luis A. Gómez, Kevin C. Dunn, and Tory M. Hagen
4.1 Abstract

Ceramides play an important role in mitochondrial function and apoptotic signaling. Accumulation of ceramides in mitochondria causes inhibition of electron transport, heightens reactive oxygen species formation, induces membrane permeability transition pores, and releases pro-apoptotic factors such as cytochrome c. Ceramides increase in response to pro-inflammatory stimuli via enhanced activity of neutral sphingomyelinase (nSMase), an enzyme that catalyzes the hydrolysis of sphingomyelin to ceramide. Furthermore, *in vitro* evidence shows that the activity of nSMase is dependent on the concentration of glutathione (GSH), which is diminished (≥ 60%) in both pro-inflammatory conditions and in aging. However, no study to date has presented evidence that mitochondrial GSH (mGSH) status can affect the activity of mitochondrial nSMase and subsequently modulate ceramide levels.

To determine the cause-and-effect relationship between mGSH and nSMase activity in mitochondria, freshly isolated rat hepatocytes were treated with (R,S)-3-hydroxy-4-pentenoic acid (3HP), a β-hydroxybutyrate analog that was previously shown to specifically deplete mGSH, and nSMase activity was determined. 3HP was used in this experiment because it is able to rapidly deplete mGSH while having minimal effect on the cytosolic pool. Results show that increasing concentrations of 3HP caused a loss of mGSH. An effective EC_{50} was established for mGSH where 3HP depleted 50% of the mGSH in less than 20 minutes (EC_{50} = 232 μM, p < 0.05). Furthermore, it was found that depletion of mGSH led to an increase in nSMase activity (24 ± 3%, p < 0.05), and an increase
in total ceramide levels (27%, p < 0.05) in mitochondria. Thus, our observations indicate that mGSH status plays a critical role in the maintenance of mitochondrial ceramide levels by regulating nSMase activity.
4.2 Introduction

Mitochondrial dysfunction plays a key role in many pro-inflammatory pathologies (10, 16-22). Diabetes, ethanol-induced liver disease, amyotrophic lateral sclerosis (ALS), and aging all share a similar phenotype whereby electron transport activity is decreased, energy metabolism is altered, reactive oxygen species (ROS) formation increases (10, 16-26), and the content of the principal low weight molecular antioxidant, glutathione (GSH), decreases by up to 60% (89, 103). The resultant decrease in mitochondrial function and antioxidant capabilities sets the stage for oxidative damage, aberrant cell signaling, increased sensitivity to external stressors, and lowers the threshold for the induction of apoptosis (24, 37, 103, 159, 160). Much research has gone into understanding the basic biochemical mechanism(s) that leads to mitochondrial dysfunction, although the answer has remained elusive.

Previously, we reported that, with age, ceramide accumulation occurs in the inner membranes of rat heart mitochondria (161). Also, in vitro evidence shows that ceramide accumulation leads to inhibition of electron transport and induces ROS formation as a consequence (74-76). It is becoming quite clear that ceramide accumulation within mitochondria (i.e. a mitochondrial “ceramidosis”) is part of the etiology of many chronic inflammatory conditions. Unfortunately, the mechanism(s) that leads to ceramidosis is not currently known. Ceramides are lipids created by four pathways: 1) de novo synthesis, which occurs in the endoplasmic reticulum, 2) re-acylation of native pools of sphingosine by reverse ceramidase activity, 3) hydrolysis of the sugar moiety from cerebrosides and
gangliosides by glucaosidases/galactosidases, and 4) the enzymatic hydrolysis of the phosphorylcholine headgroup of native sphingomyelin pools by sphingomyelinases (see Figure 1.3, Chapter 1). Mitochondria do not contain the necessary machinery for de novo synthesis of ceramides (87). We found that sphingosine levels are not sufficiently high enough to achieve the noted accumulation of ceramides. Moreover, glycosphingolipids have not been reported to be in the mitochondria at high concentrations, and we found no trace of glucosyl or glycosylceramide in mitochondria. This suggests that neither reverse ceramidase activity nor glucosidase/glycosidase activity could be responsible for the accumulations noted. However, the presence of the pH neutral optima isoform of sphingomyelinase (nSMase) has been reported in mitochondria (87). This raises the question whether mitochondrial ceramidosis seen in aging and other pro-inflammatory disease states is caused by an activation of the enzyme nSMase. This is further supported by the work of Hannun and colleagues showing that nSMase activity is non-competitively inhibited by physiological concentrations (1 to 10 mM) of GSH (89, 90). As we have previously reported, there is a loss in mGSH with age (161) and we hypothesize that its depletion causes an up-regulation of nSMase activity.

In this study, we examine whether modulation of mGSH levels can alter the enzymatic activity of nSMase in mitochondria. Freshly isolated hepatocytes from male F344 rats were used. The choice to use hepatocytes in lieu of myocytes was based on three factors: 1) the yield of cardiomyocytes from a single rat heart is insufficient to carry out the development phase of this work: 2) hepatocytes
from F344 rats have a well-characterized stress response mechanism including sphingomyelinases (38, 40); and 3) large quantities of functional, and relatively pure, mitochondria can be easily obtained from their hepatocytes. To elucidate the effect of mGSH status on nSMase activity, we specifically depleted mGSH by using \((R,S)-3\)-hydroxy-4-pentenoic acid (3HP). This ketone body analog is a substrate of the mitochondrial enzyme, 3-hydroxybutanoate dehydrogenase, which converts 3HP to the Michael acceptor 3-oxo-4-pentenoic acid (3OP) (102, 162, 163). The enone intermediate readily binds GSH and causes its depletion within mitochondria, without large alterations of the cytosolic GSH.

The findings reported here suggest a biochemical mechanism for the accumulation of ceramide within mitochondria as a consequence of alteration of mGSH levels. Furthermore, this ceramide accumulation appears to be caused by the enzymatic hydrolysis of sphingomyelin by nSMase, which in turn, is activated by a decrease in mGSH. Therefore, this mechanism has the potential to explain an important aspect in the etiology of pro-inflammatory pathologies and aging.
4.3 Materials and Methods

Reagents

Dansyl chloride, γ-glutamyl-glutamate, reduced and oxidized glutathione, Genistein, Triton X-100, and Tween 20 were from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (fraction V, fatty acid free) was obtained from Calbiochem (EMD Biosciences, La Jolla, CA); digitonin was from Acros (Pittsburg, PA), 3-hydroxy-4-pentenoic acid was purchased from Epsilon Chimie (Brest-Guipavas, France), while purified ceramide standards were purchased from Avanti Polar Lipids (Alabaster, AL).

Ethical treatment of vertebrate animals

Fischer 344 rats (male, 4-6 months old) were obtained from the National Institute on Aging animal colonies. Animals were housed in approved facilities and maintained by the Oregon State University Laboratory Animal Resources Center. All animal procedures were performed in accordance with OSU guidelines for animal experimentation and approved by the institutional animal care and use committee (IACUC).
Depletion of mitochondrial glutathione using 3HP

Hepatocytes were isolated by collagenase perfusion as previously described by Smith et al. (164). Isolated hepatocytes in suspension were incubated in Williams E media (supplemented with 0.25 M dexamethasone, 10 mg/ml insulin, 1000 units penicillin, 0.1 mg streptomycin, 0.25 μg amphotericin B, 200 mM L-glutamine, and 5% fetal bovine serum) containing 3HP at the concentrations indicated in the text, at 37°C for 4 hours. Hepatocytes were kept in suspension by rocking gently. Samples for GSH analysis were immediately stored in PCA/DTPA in an effort to limit any ex vivo oxidation. All other samples were stored in an isotonic buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM tris-base, 5 mM KH₂PO₄, pH 7.2). GSH was monitored using dansyl chloride as previously described (152). nSMase activity was determined by quantifying the hydrolysis of a fluorescently labeled sphingomyelin as previously described (161). As a control, GW4869 was used to inhibit the activity of nSMase.

Mitochondrial isolation

Mitochondria were isolated using differential centrifugation. Briefly, hepatocytes were suspended in a mild lysis buffer (250 mM D-mannitol, 0.5 mM EGTA, 5 mM Tris-HCl, 10 mM triethanolamine, supplemented with 0.2% bovine serum albumin and 0.1 mg/ml digitonin) on ice for 10 minutes followed
disruption using a Dounce homogenizer (MitoSciences; Eugene, OR). The homogenate was centrifuged at 600 x g for 5 minutes. The supernatant was saved and the pellet re-suspended in buffer (250 mM D-mannitol, 0.5 mM EGTA, 5 mM Tris-HCl, 10 mM triethanolamine, supplemented with 0.2% bovine serum albumin) and disrupted further with the Dounce homogenizer. The homogenate was spun again for 5 minutes at 600 x g. The pellet was discarded and the supernatant was combined with the previous one and centrifuged for 10 minutes at 10,000 x g, yielding a crude mitochondrial fraction. All steps of the isolation were performed on ice or at 4°C. Protein values were determined using the BCA protein assay kit (Thermo Scientific; Rockford, IL).

**Lipid Extraction**

All samples were prepared as described in Merrill et al. (134). Briefly, internal standard (IS) (25 μl, Avanti Polar Lipids, Alabaster, AL), composed of a mixture of synthetic sphingolipids not found in mammals, was added to mitochondria (1 mg protein) and extracted with 1.5 ml of chloroform:methanol (1:2). After a two hour incubation at 48°C, 75 μl of 1 M KOH in methanol was added and the samples were incubated at 37°C for an additional hour to remove free fatty acids and triacylglycerols. The pH was then neutralized with glacial acetic acid. Phase separation was achieved by adding chloroform (2 ml) and H₂O (4 ml). The chloroform layer was aspirated and dried under N₂. The sample was
reconstituted in 200 μl chloroform:methanol (3:1) and diluted 1:4 with acetonitrile:methanol:acetic acid (97:2:1) containing 5 mM ammonium acetate.

**LC Tandem Mass Spectrometry**

HPLC was carried out using a binary pump system with an auto-injector, degasser, and column oven (Shimadzu, Columbia, MD, USA). A Supelco Discovery column (2 mm x 50 mm) was used along with Security-Guard NH₂ guard cartridges (4 x 2 mm) (Phenomenex, Torrance, CA, USA). The sample chamber and the column temperature were controlled at 10°C and 35°C, respectively. The mobile phase contained 0.2% (v/v) formic acid and 10 mM ammonium acetate with a gradient of solvent A (methanol:water, 60:40) and solvent B (methanol:chloroform, 60:40) at a flow rate of 300 μl per minute. The column was equilibrated for 3 minutes at 100% A before sample injection (typically 5 μl). Then, 100% A was maintained for one minute, followed by a linear increase to 40% B to 8.0 minutes, next a linear increase to 70% B to 13 minutes; 70% B was then maintained for the remainder of the 20 minute run.

The HPLC system was coupled through a TurboIon Spray source to a triple-quadrupole mass spectrometer operated in positive mode (Applied Biosystems/MDS Sciex API 3000, Foster City, CA, USA). Optimizations of the mass spectrometer were done manually. Analytes were detected using Multiple
Reaction Monitoring (MRM) mode, which selectively detects fragment ions from the collision-induced dissociation (CID) of the parent molecular ion. Figure 1.1 (see chapter 1) shows the structure of C_{16}-ceramide and the proposed cleavage point that creates the major fragment ion 264 m/z, which is characteristic for ceramides.

Breakdown of ceramide to the 264 m/z CID product was highly reproducible, allowing quantification by comparison to known purified standards. A standard curve using purified synthetic C_{16}-ceramide yielded a linear range from 3 fmol with the highest standard of 625 fmol. The limit of quantitation was determined by the lowest standard having a peak height being no less than 10 times the signal-to-noise ratio. The sensitivity of this method was considered robust, with an effective range of quantitation spanning at least 3 orders of magnitude. Of note, synthetic standard for C_{22}-ceramide is not currently available; all quantification of this particular ceramide used the standard curve created for C_{24}-ceramide.

Statistics

Data are presented as mean ± SEM. Samples were assessed for statistical significance using Student’s t test. A p value <0.05 was considered statistically significant.
4.4 Results

*Depletion of mitochondrial glutathione content by 3-hydroxy-4-pentenoate*

Control mitochondria contained 19.61 ± 1.067 nmols GSH/mg protein. 3HP treatment of rat hepatocytes resulted in a concentration-dependent depletion of mGSH (Figure 4.1). The maximum depletion of mGSH occurred in less than 30 minutes and remained stable for the duration of the incubation (data not shown). The concentrations used and the depletion achieved is in keeping with previous reports (38, 102, 162). Treatment with 3HP led to a 16% decrease in cytosolic GSH content (n = 1), which is in keeping with previous reports (38, 102) and may reflect diminished mGSH pool as mitochondria comprise approximately 18% of hepatocytes volume.

Treating hepatocytes with 3HP up to 1 mM did not result in any decrease in cell viability over the 4 hour experiment as measured by trypan blue exclusion. 3HP-dependent depletion of mGSH occurred with concentrations as low as 50 μM while 1 mM 3HP yielded a near complete removal of mGSH (Figure 4.1A). Analyzing the concentration-dependent mGSH loss revealed an EC₅₀ of 232 μM. Based on this analysis, we chose a concentration of 250 μM 3HP for our experiments as using this concentration results in mGSH which approximates the loss in this tripeptide evident in aging hepatic steatosis (18, 114, 165).
Glutathione dependence of mitochondrial neutral sphingomyelinase activity

Increasing amounts of 3HP given to hepatocytes resulted in a step-wise increase in mitochondrial nSMase activity (Figure 4.2A). Ultimately, nSMase activity increased by 21% (1000 ± 28.4 vs. 819 ± 7.5 pmol/mg protein in treated and control groups, respectively) after a 4 hour incubation of hepatocytes with 250 μM 3HP. In order to verify that the sphingomyelinase activity observed was from the neutral isoform, mitochondria were incubated with GW4869, a specific inhibitor of nSMase. GW4869 (1 μM) markedly inhibited nSMase activity such that it was no longer detectable (Figure 4.2B), suggesting that the sphingomyelinase activity found in hepatic mitochondria is from the pH neutral isoform.

In order to verify that alteration of GSH levels is the mechanism for regulation of nSMase activity, mitochondria isolated from hepatocytes that were treated with or without 3HP, were solubilized and repleted with increasing concentrations of GSH. GSH addition caused a rapid pepletion of mGSH levels (data not shown). Consequently, nSMase activity was dimished in an inverse manner with respect to mGSH repletion in both 3HP treated and un-treated groups (Figure 4.2C). As a whole, this evidence supports our hypothesis that the activity of the nSMase found in mitochondria can be regulated by physiological levels of mGSH.
Depletion of mitochondrial glutathione results in the accumulation of ceramide

As in the heart, mitochondria from rat hepatocytes contain a small group of ceramides with acyl side-chains ranging from 14 to 24 carbons and are mostly saturated (Table 4.1). Furthermore, the concentrations of individual homologs are maintained within a narrow range. C_{24}\text{-ceramide} was found in the greatest abundance followed by C_{16}\text{-}, C_{24:1}\text{-}, and C_{22}\text{-ceramide}. Unlike the heart, hepatic mitochondria contain an additional homolog, C_{14}\text{-ceramide}, and two additional mono-unsaturated species, C_{18:1}\text{- and C}_{22:1}\text{-ceramide}.

Depletion of mGSH by a four-hour treatment with 250 \( \mu \text{M} \) 3HP resulted in an increase in C_{18}\text{-, C}_{20} \text{ and C}_{22}\text{-ceramide [which were by 44.6, 32.5, and 30.7\%, above their respective controls (p < 0.050)]. Lower mGSH also tended to increase all other ceramide homologs as well. On a total ceramide basis, there was an increase of 27\% over the controls (p < 0.05) (Figure 4.3).
Figure 4.1 Mitochondrial glutathione content of rat hepatocytes following treatment with 3-hydroxy-4-pentenoate (3HP). Hepatocytes were treated with increasing concentrations of 3HP for four hours followed by mitochondrial isolation (See methods). GSH was labeled using dansyl chloride and monitored by HPLC coupled to fluorescence detection. (A) 3HP treatment results in a concentration-dependent depletion of mGSH (EC₅₀ = 232 μM). (B) Treatment of hepatocytes with the nSMase inhibitor, GW4869, has no effect on 3HP-dependent mGSH depletion. Data represent means ± SEM, n = 3.
Figure 4.2 nSMase activity is significantly increased by GSH depletion. nSmase activity was monitored in 3HP-treated mitochondria using a fluorescently labeled ceramide analog as the substrate followed by HPLC coupled to fluorescence detection of the products. (A) 3HP treatment caused a concentration-dependent increase in nSMase activity. (B) nSMase increase upon 3HP treatment (250μM) is shown with respect to controls; the nSmase inhibitor GW4869 (10 μM) was used to inhibit activity. (C) Mitochondrial isolated from hepatocytes treated with and without 250 μM 3HP were solubilized, pre-incubated for 15 minutes with GSH at noted concentrations, and nSMase was then assessed. Results show a concentration-dependent inhibition of nSmas activity. Data represent means ± SEM, n = 6, and asterisk (*) denotes a significant difference from controls, p < 0.05, using a one-way analysis of variance with a Tukey post-hoc test.
Figure 4.3 Depletion of glutathione results in total ceramide accumulation. Rat hepatocytes were treated with 250 μM 3HP for 4 hours followed by mitochondrial isolation (see methods). Total ceramides were measured by LC-MS/MS. Results show that 3HP-dependent depletion of mGSH causes a significant increase in total ceramides. Data represent means ± SEM, n = 5, and asterisk (*) denotes a significant difference from controls, p < 0.05, using Student’s t test.
Table 4.1 Effect of GSH depletion on individual ceramide species.

<table>
<thead>
<tr>
<th>Ceramide Species</th>
<th>Control</th>
<th>+3HP</th>
<th>Δ% from Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein</td>
<td>pmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>C_{14}-ceramide</td>
<td>45.3 ± 6.9</td>
<td>49.3 ± 9.0</td>
<td>8.8</td>
</tr>
<tr>
<td>C_{16}-ceramide</td>
<td>293.4 ± 23.1</td>
<td>393.2 ± 49.7</td>
<td>34.0</td>
</tr>
<tr>
<td>C_{18}-ceramide</td>
<td>27.3 ± 0.7</td>
<td>39.5 ± 1.2*</td>
<td>44.6</td>
</tr>
<tr>
<td>C_{20}-ceramide</td>
<td>19.3 ± 0.6</td>
<td>25.6 ± 1.6*</td>
<td>32.5</td>
</tr>
<tr>
<td>C_{22}-ceramide</td>
<td>231.0 ± 10.0</td>
<td>302.0 ± 22.2*</td>
<td>30.7</td>
</tr>
<tr>
<td>C_{24}-ceramide</td>
<td>924.1 ± 121.5</td>
<td>1061.0 ± 114.6</td>
<td>14.8</td>
</tr>
<tr>
<td>C_{18:1}-ceramide</td>
<td>19.2 ± 0.8</td>
<td>23.8 ± 1.7</td>
<td>24.1</td>
</tr>
<tr>
<td>C_{22:1}-ceramide</td>
<td>19.7 ± 1.9</td>
<td>26.0 ± 2.3</td>
<td>31.5</td>
</tr>
<tr>
<td>C_{24:1}-ceramide</td>
<td>305.2 ± 11.6</td>
<td>385.8 ± 36.9</td>
<td>26.4</td>
</tr>
</tbody>
</table>

All data are represented as means ± SEM, n = 5.

*p < 0.05 vs. control
4.5 Discussion

To the best of our knowledge, this is one of the few studies to address the effect of mitochondrial-specific glutathione depletion on an agent that causes mitochondrial dysfunction and induces apoptosis. Previous attempts to understand the role of GSH utilized chemical agents such as buthionine sulfoximine ($\gamma$-glutamylcysteine synthetase inhibitor) which do not readily affect the mitochondrial pool of GSH (166, 167). The major problem with experiments that deplete cellular glutathione is that nSMase is highly abundant on the cytosolic side of the plasma membrane (168). Depletion of total GSH would quickly activate nSMase and induce whole-cell stress signaling, which includes signaling to the mitochondria. Thus, one advantage of the present work is that our results are based on the specific depletion of mGSH with little alteration of cytosolic GSH.

One possible confounding factor in the interpretation of the results is the possibility of a direct interaction between the 3-oxo-4-pentenoate (3OP) intermediate and nSMase. This interaction is not likely as, 3HP itself does not interact with nSMase (Data not shown). Also, Lui et al. showed that inhibition of nSMase by glutathione is a highly specific reaction involving the $\gamma$-glutamylcysteine moiety of GSH, and is not dependent on the thiol functional group or the redox state of GSH (90). In addition, this study showed that repletion of GSH to 3HP treated mitochondria reversed the inhibition and resulted in a similar inhibition of nSMase activity as that found in the mitochondria not treated with
3HP (Figure 4.2C). 3OP is presumably the highly reactive intermediate and Michael acceptor that ultimately causes the GSH adduction and depletion. 3OP addition to nSMase is also possible, which would result in a non-reversible covalent modification of the protein. If this were occurring, GSH repletion would not be expected to be able to reverse this modification. Because GSH repletion was able to reverse the 3HP-dependent nSmase activation, it is not likely that a direct interaction between 3OP and nSMase is occurring.

The increases in nSmase activity and ceramide levels found in this study were modest. This raises the question as to whether mitochondrial function and/or cellular signaling could be affected. In this respect, several lines of evidence support the concept that the alterations reported in this work are consequential. First, depletion of mGSH content in MCF-7 and MDA-MB-231 cells leads to an increased sensitivity to ceramide-dependent apoptosis (169). Second, García-Ruiz et al. showed that incubation of hepatocytes with 3HP caused a dramatic increase in sensitivity to TNF-α (38). It is interesting to note that in this work that 3HP-treatment of hepatocytes resulted in a trend towards an increase in apoptosis, while co-incubation with TNF-a led to massive cell death. While little work has been done to understand the interaction of mitochondrial glutathione, ceramides and cell health, these studies support our hypothesis that depletion of mGSH results in mitochondrial dysfunction and a decreased threshold for apoptosis.

One of the interesting findings reported here, is that there was a general increase in all ceramide isoforms, but with each homolog increasing by different amounts (Table 4.1). This asymmetric increase is reminiscent of that seen in
mitochondria from aged rat hearts (see chapter 3). One possible explanation is that nSMase does not discriminate between the different sphingomyelin substrates. In fact, analysis of the ratio of each acyl chain length species of sphingomyelin to its corresponding ceramide reveals that the percent change matches the sphingomyelin-to-ceramide ratio (Supplemental Figure 2, Appendix). This suggests that nSMase does not distinguish between different homologs of sphingomyelin. This also suggests that liver mitochondria lack the ceramide distribution asymmetry previously observed in cardiac mitochondria (149).

In conclusion, the results of this study are important as they show a biochemical mechanism for mitochondrial ceramide accumulation due to loss in mGSH. We also show that it is possible that the loss in glutathione levels may be upstream to ceramide accumulation. This is supported by the work of Pehar et al., whereby they showed a critical link between GSH loss, mitochondrial nSMase activation, and ceramide accumulation in a model for ALS. Interestingly, by activating Nrf2-mediated antioxidant responses and restoring mGSH, they could completely abrogate the ceramide accumulation and apoptosis (159).

**Acknowledgments and notice of grant support**

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Chapter 5

(R)-α-Lipoic Acid Treatment Restores Ceramide Balance in Aging Rat Cardiac Mitochondria

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Data from this chapter are excerpts from the published article entitled (R)-α-Lipoic Acid Treatment Restores Ceramide Balance in Aging Rat Cardiac Mitochondria.


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5.1 Abstract

Inflammation results in heightened mitochondrial ceramide levels, which cause electron transport chain dysfunction, elevate reactive oxygen species, and increase apoptosis. As mitochondria in aged hearts also display many of these characteristics, we hypothesized that mitochondrial decay stems partly from an age-related accumulation in mitochondrial ceramides (ceramidosis) that heretofore has not been recognized for the heart. Ceramide levels increased by 32% with age and three ceramide isoforms, previously found to reside primarily in the inner mitochondrial membrane (e.g. C_{16}-, C_{18}-, and C_{24:1}-ceramide), caused this increase. The evident ceramidosis may stem from enhanced hydrolysis of sphingomyelin, as neutral sphingomyelinase (nSMase) activity doubles with age but with no attendant change in ceramidase activity. Because (R)-α-lipoic acid (LA) improves many parameters of cardiac mitochondrial decay in aging and lowers ceramide levels in vascular endothelial cells, we hypothesized that LA may limit cardiac ceramidosis and thereby improve mitochondrial function.

Mitochondria were isolated from young (4-6 mo) and old (26-28 mo) F344 male rats fed LA (0.2% w/w) for two weeks and analyzed for ceramides by LC-MS/MS. Results showed that LA-treatment to old rats reversed the age-associated decline in glutathione levels and concomitantly improved Complex IV activity. This improvement was associated with lower nSMase activity and a remediation in mitochondrial ceramide levels. In summary, LA treatment lowers ceramide levels to that seen in young rat heart mitochondria and restores Complex IV activity which otherwise declines with age.
5.2 Introduction

Mitochondria from the aging heart are in a constant state of low-level inflammation (18, 112, 164). This manifests as decreased glutathione levels, inhibition of electron transport, altered energy metabolism, increased reactive oxygen species formation, and increased oxidative damage. In addition to these well-known hallmarks, we previously showed that cardiac mitochondria from aged rats have increased levels of the pro-inflammatory signaling lipid, ceramide (161). In vitro experiments using ceramide analogs show that the accumulation of ceramide in the mitochondria yields a mitochondrial dysfunction that is strikingly similar to that seen in aging (74, 76, 77, 125, 148, 170), suggesting that ceramide accumulation may be an important part of the mitochondrial dysfunction seen with age.

Ceramides are synthesized either by de novo synthesis in the endoplasmic reticulum or they are created within the target membranes, including mitochondria, by a family of enzymes called sphingomyelinases, which create ceramide by hydrolyzing the phosphorylcholine headgroup from native sphingomyelin. Increases in the activities of neutral and acidic isoforms of sphingomyelinase (nSMase and aSMase, respectively) activity have been noted to occur during various stress responses (101, 159, 168), but only the neutral isoform has been found in mitochondria (141, 171). The activity of nSMase is regulated by glutathione, which is depleted by 60% in cardiac mitochondria with age (18, 172). Mechanistically, this provides the possibility that, by restoring mitochondrial glutathione (mGSH), we can stop the aberrant increase in nSMase
activity, thus abolishing the ceramidosis. Treatment with the anti-inflammatory agent \((R)-\alpha\)-lipoic acid (LA) has been shown to cause the reversal of age-related mGSH decline \((114, 164, 172)\).

LA is a naturally occurring dithiol compound synthesized in mitochondria and is an essential cofactor for alpha-ketoacid dehydrogenases. LA-supplementation has been used for the treatment of an array of pro-inflammatory conditions and neurological disorders such as atherosclerosis \((173)\), diabetes \((174)\), multiple sclerosis \((108)\), and ischemia/reperfusion injury \((110)\). LA acts as a potent anti-inflammatory agent at pharmacological doses \((106, 108, 109, 132, 172)\). Supplementation with LA has been shown in many reports to reduce aberrant stress signaling, restore proper metabolism, and bolster antioxidant responses. Moreover, we recently reported that when old rats were treated with LA, age-associated increases in nSMase activity were limited and ceramide imbalance in aortic endothelia was remediated \([160]\). We have also previously shown that LA lowers indices of mitochondrial dysfunction \((115-117)\), thus providing a rationale that LA may reverse at least certain aspects of mitochondrial decay by opposing ceramidosis. Therefore, the goal of this study was to determine whether LA could remediate the age-related ceramide accumulation found in cardiac mitochondria, thereby ameliorating the mitochondrial aging phenotype.
5.3 Materials and Methods

Chemicals and antibodies

Digitonin, genistein, Subtilisin A (type VIII), Triton X-100, and Tween 20 were from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (fraction V, fatty acid free) was obtained from EMD Biosciences (La Jolla, CA). Purified ceramide standards were purchased from Avanti Polar Lipids (Alabaster, AL). NBD-sphingomyelin and NBD-ceramide were purchased from Life Technologies (Carlsbad, CA). Rabbit polyclonal antibody to the voltage-dependent anion channel protein (VDAC) and mouse monoclonal antibody to protein disulfide isomerase (PDI) were purchased from Abcam, Inc. (Cambridge, MA). All other compounds were reagent grade or of the highest purity obtainable.

Ethical treatment of vertebrate animals

Young (4-6 mo) and old (26-28 mo) Fischer 344 male rats were obtained from the National Institute on Aging animal colonies. Animals were housed in approved facilities and maintained by the Oregon State University Laboratory Animal Resources Center. All animal procedures were performed in accordance with OSU guidelines for animal experimentation and approved by the institutional animal care and use committee (IACUC).
Lipoic acid supplementation

Rats were fed an AIN-93M diet (Dyets Inc., Bethlehem, PA) ± 0.2% (w/w) LA (MAK Wood Inc., Grafton, WI) for two weeks prior to sacrifice. Because a two-week LA-treatment results in a mild hypophagia, animals were pair-fed. LA-treated animals were fed *ad libitum* and food consumption was measured every 24 hours. Rats on the unsupplemented diet were given the same amount of food as the supplemented ones consumed the previous day. By this staggered protocol of pair-feeding, the possibility of a caloric intake difference affecting the data was eliminated. Although food consumption decreased over the course of the two-week treatment (18.75 g and 23.69 g consumed on the first day of treatment decreased to 13.18 and 16.23 g on day thirteen for young and old rats, respectively), all animals maintained a consistent body weight.

Animals were sacrificed between 8:00 AM and 12:00 PM. Rats were first anesthetized by diethylether inhalation, and heparin [0.2% (w/v); 1.0 ml/kg b.w.] was injected into the iliac artery to prevent blood clotting. The animal was then sacrificed by cutting through the diaphragm and exposing the heart. The heart was perfused with ice-cold phosphate buffered saline, pH 7.4, immediately excised, and placed in ice-cold buffer for a few minutes until mitochondrial isolation.

Mitochondrial isolation

Cardiac mitochondria were isolated using differential centrifugation as described by Palmer *et al.* (27) with modifications as in Monette *et al.* (149). This
procedure resulted in an enriched interfibrillar mitochondrial fraction. All steps of the isolation were performed on ice or at 4°C. Protein values were determined using the BCA protein assay kit (Thermo Scientific; Rockford, IL).

**Inner mitochondrial membrane (IMM) isolation**

The outer mitochondrial membrane (OMM) was selectively removed using digitonin (131). Six mg/ml digitonin at 37°C in isotonic buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM tris-HCl, 5 mM KH2PO4, pH 7.2) was optimal for removing the OMM. This procedure resulted in a highly purified IMM fraction with less than 2% contamination from OMM as determined by immunoblotting for VDAC. The IMM could not be further purified by Percoll density centrifugation as yields were too low to allow for LC-MS/MS analysis.

**Mitochondrial Complex IV activity**

Cytochrome c oxidase (Complex IV) activity was measured using a commercially available kit from Sigma-Aldrich that follows the oxidation of cytochrome c.
Activity assay for neutral sphingomyelinase and ceramidase

Fluorescently-labeled sphingomyelin and ceramide (NBD-sphingomyelin and NBD-ceramide, respectively) were used as substrates to determine the activities of ceramide metabolizing enzymes by the method of Lightle et al. (151).

Measurement of glutathione (GSH)

GSH was conjugated to dansyl chloride and measured by using HPLC and fluorescence detection as described by Dixon et al. (152).

Lipid extraction

All samples were prepared as in Merrill et al. (134) except that samples were not saponified in KOH.

LC-tandem mass spectrometry

Lipids were separated by HPLC using a Supelco Discovery column (2 mm x 50 mm; Sigma-Aldrich). The flow rate was set at 300 µl per minute. Mobile phase A contained methanol:water (60:40) while mobile phase B was composed of methanol:chloroform (60:40). Both solvents contained 0.2% (v/v) formic acid and 10 mM ammonium acetate. The pump schedule was as follows: the column was pre-equilibrated at 100% mobile phase A followed by sample injection (5 µl);
100% mobile phase A was maintained for one minute, followed by a linear increase to 40% mobile phase B over a 7 minute period; followed by a linear increase to 70% mobile phase B over the next 6 minutes; 70% mobile phase B was maintained for the remainder (6 minutes) of the 20 minute run.

Analytes were detected on a triple-quadrupole mass spectrometer operated in positive mode (Applied Biosystems/MDS Sciex, API 3000) using multiple reaction monitoring, which selectively detects fragment ions from the collision-induced dissociation of the parent molecular ion. For a list of molecular ion transitions, please see Monette et al. (149). Quantitation was based on comparison to synthetic sphingolipid standards.

**Statistics**

Data are presented as means ± SEM. Samples were assessed for statistical significance using a one-way ANOVA test. Multiple comparisons were made using a Tukey’s post hoc test or the Student’s t-test. A p value ≤ 0.05 was considered statistically significant.
5.4 Results

Lipoic acid supplementation reverses age-related mitochondrial ceramide accumulation

In young rats, LA treatment yielded no apparent changes in overall levels of mitochondrial ceramides (Figure 5.1). Furthermore, only modest changes in ceramide isoforms were noted when comparing young LA-treated animals to the age-matched controls (Table 5.1). Specifically, $C_{22}$-ceramide increased by 14.2%, while $C_{24:1}$-ceramide decreased by 15%. All other species were altered by less than 10%. Overall, this suggests that treatment with LA had minimal effect on mitochondrial ceramides from young animals, indicating that LA does not modulate ceramide metabolism directly.

For old rats, however, LA lowered general cardiac ceramide levels such that they were no longer different than that seen in hearts from young animals (Figure 5.1). When specific ceramide isoforms were examined, we observed that LA treatment caused a significant decrease in $C_{18}$-ceramide ($p < 0.05$) when compared to old control animals (Table 5.1). All other species, though not reaching statistical significance, showed a trend for a decrease of approximately 30%, with the exception of $C_{22}$-ceramide (Table 5.1). The near uniform decrease evident in all species of ceramide found in LA-treated old animals suggests that the mechanism of LA-dependent mitochondrial ceramide reduction occurs, not by lowering specific ceramide species, but by decreasing ceramides in general.
Figure 5.1 LA treatment decreases mitochondrial ceramides. Young and old rats were fed (R)-α-lipoic acid (LA; 0.2% [w/w]) or a control diet for two weeks. Mitochondria were isolated, lipids extracted and analyzed by LC-MS/MS. Total ceramide was increased in cardiac mitochondria from aged rats; LA restored ceramides to levels seen in young animals but resulted in no alteration of ceramide levels in young rats. Data represent the means ± SEM, n = 4; an asterisk (*) denotes a significant difference by Student’s t-test between old and young control animals, p < 0.03.
Table 5.1 Cardiac mitochondrial ceramide levels with and without lipoic acid supplementation

<table>
<thead>
<tr>
<th>Ceramide Species</th>
<th>Young Control</th>
<th>Old Control</th>
<th>Young +LA</th>
<th>Old +LA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein</td>
<td>pmol/mg protein</td>
<td>Δ% from Young Control</td>
<td>pmol/mg protein</td>
</tr>
<tr>
<td>C_{16}\text{-ceramide}</td>
<td>7.4 ± 0.6</td>
<td>12.7 ± 1.9*</td>
<td>72.3</td>
<td>8.0 ± 0.6</td>
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<tr>
<td>C_{18}\text{-ceramide}</td>
<td>7.8 ± 1.3</td>
<td>13.5 ± 1.2*</td>
<td>73.4</td>
<td>8.2 ± 1.0</td>
</tr>
<tr>
<td>C_{20}\text{-ceramide}</td>
<td>12.5 ± 1.5</td>
<td>14.8 ± 1.1</td>
<td>18.1</td>
<td>13.2 ± 2.2</td>
</tr>
<tr>
<td>C_{22}\text{-ceramide}</td>
<td>27.7 ± 2.6</td>
<td>30.0 ± 0.9</td>
<td>8.0</td>
<td>31.7 ± 2.7</td>
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<tr>
<td>C_{24}\text{-ceramide}</td>
<td>40.6 ± 6.4</td>
<td>48.8 ± 5.6</td>
<td>20.0</td>
<td>38.2 ± 3.1</td>
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<tr>
<td>C_{24:1}\text{-ceramide}</td>
<td>10.9 ± 1.8</td>
<td>19.4 ± 4.2</td>
<td>77.7</td>
<td>9.3 ± 1.4</td>
</tr>
</tbody>
</table>

All data are represented as means ± SEM, n = 4.
* p < 0.05 vs. young control
* p < 0.05 vs. old control
+LA, denotes the lipoic acid supplemented groups
Figure 5.2 LA treatment restores Complex IV activity in cardiac mitochondria from aged animals. Isolated cardiac mitochondria from LA-supplemented or non-supplemented rats were assayed for Complex IV activity. Enzymatic activity declined with age and was restored by LA to the levels seen in young animals. Data represent the means ± SEM, n = 4; an asterisk (*) denotes a significant difference from old controls, p ≤ 0.05.
*Lipoic acid treatment reverses age-related deficiency in Complex IV activity.*

Mitochondria from young LA-supplemented rats exhibited no treatment-related changes in Complex IV activity when compared to mitochondria from non-supplemented animals (Figure 5.2). This indicates that LA does not directly modify cytochrome oxidase *per se*. However, for old rats, LA reversed the loss of Complex IV function to a point where its activity was no longer different from young animals (Figure 5.2). We therefore conclude that LA modulates Complex IV activity only in mitochondria from aged tissue where elevated IMM ceramides are evident.

*Lipoic acid treatment restores proper neutral sphingomyelinase activity in mitochondria from old animals.*

While there is a positive association between LA-induced reversal of ceramide accumulation and improved ETC activity, these results do not provide a potential mechanism by which LA causes these remediative effects. As pro-inflammatory stimuli induce ceramidosis by activating sphingomyelinas, we hypothesized that LA may at least partially work through modulating nSMase activity to limit age-related increases in mitochondrial ceramides. Mitochondrial nSMase activity significantly increased by 103% with age (*p* < 0.05) (Figure 5.3); however, mitochondria from LA-supplemented old rats displayed no age-related elevations in nSMase activity. In keeping with its action on ceramide levels, LA did not alter nSMase activity in young rats.
Figure 5.3 LA restores neutral sphingomyelinase (nSMase) activity in mitochondria from old animals to youthful levels. Cardiac mitochondria from young and old rats fed LA or the control diet for two weeks were assayed for nSMase activity. nSMase activity significantly increased with age and was restored to youthful levels by LA treatment. Data represent the means ± SEM, n = 4; an asterisk (*) denotes a significant difference, p < 0.01.
Because mitochondria reportedly also contain ceramidases, which catabolize ceramide to sphingomyelin (175), further experiments were performed to determine whether an age-related loss of ceramidase activity might also contribute to the mechanism by which LA reverses age-dependent mitochondrial ceramidosis. Even though cardiac mitochondrial ceramidase was detectable, no age-associated change in its activity was noted (data not shown). Furthermore, LA supplementation had no effect on ceramidase activity in either young or old animals. This indicates that aging causes an imbalance between elevated nSMase-induced ceramide production and ceramidase-mediated catabolism, which could contribute to the ceramide accumulation observed in aging rat heart mitochondria. Combined, these results suggest that pharmacological doses of LA reverse ceramide accumulation in old rat heart mitochondria, potentially through limiting elevations in nSMase activity.

**Lipoic acid treatment partially restores the deficit in cardiac mitochondrial glutathione levels evident with age**

Because previous reports have shown nSMase activity is inversely proportional to GSH status (89, 90, 101) and GSH levels decline markedly in the aging heart, we hypothesized that the LA-mediated improvement in mitochondrial ceramide status was through restoring mitochondrial GSH levels (17, 142). As anticipated, LA-treatment did not alter GSH levels in young versus controls. However, LA reversed the 43% age-associated decline in mitochondrial GSH
Figure 5.4 LA markedly increases mitochondrial glutathione levels that otherwise decrease with age. GSH levels were monitored in mitochondria from young and old rats fed LA or the control diet. GSH content significantly decreased with age and was restored to youthful levels by LA treatment. Data represent the means ± SEM, young, n = 3; old, n = 4; an asterisk (*) denotes a significant difference, p ≤ 0.05.
levels, such that the loss was no longer statistically different than in young untreated rats (Figure 5.4). These results indicate that the LA-mediated reduction in mitochondrial ceramidosis may ultimately stem from its means of controlling nSMase activity by maintenance of mitochondrial GSH.
5.5 Discussion

The major highlight of this report is the identification of LA as an agent to restore both mitochondrial ceramide levels and Complex IV activity to that seen in mitochondria in young rats. This further supports the pharmacological mechanism presented in chapter four, by which LA reverses the evident ceramidosis and ETC dysfunction by limiting age-dependent increases in mitochondrial nSMase activity through restoration of GSH levels. We previously showed that the GSH redox status of the myocardium and cardiac interfibrillar mitochondria is altered with age, and that LA treatment corrects these changes (114, 164, 176-178). This is consistent with our current results showing that feeding LA markedly improved the mitochondrial GSH status in old rat hearts, strengthening the concept that age-associated decline in mitochondrial GSH may be responsible for the elevated nSMase activity.

Our results clearly showed that LA did not affect mitochondrial ceramide levels in young rats, but merely restored ceramide values in aged animals to the norm. Thus, its general use as a prophylactic to prevent conditions that may lead to mitochondrial ceramidosis would appear to have few adverse consequences in young healthy subjects. This is in keeping with results from human clinical trials where the use of LA, even at relatively high pharmacological doses (1800 mg/day), resulted in only few side-effects, the predominant one being gastric upset (174). On a cellular level, this also suggests that LA would not inappropriately cause loss in membrane ceramides. Maintenance of ceramides at normal levels is vital to preserve their role in membrane fluidity, and as a
modulator of many kinases and phosphatases (82). Thus, LA may be an appropriate adjunct to limit age-related mitochondrial ceramidosis and the adverse cardiac effects that its accumulation causes.
Chapter 6

General Conclusions
6.1 General Conclusions

With the “baby boomer generation” retiring, the United States is about to experience a dramatic increase in the elderly population (1). Age is the primary risk factor for, heart-related diseases and disorders such cardiovascular diseases and heart failure (HF) (7). So, with this “age wave” will come a large increase in the incidence of elderly with some form of heart related disorder. Therefore, understanding the mechanisms of aging has never been more important than it is right now. Unfortunately, over a century of research has primarily yielded information concerning the aging phenotype and not the mechanism(s) associated with deficits of daily living. Current therapies to deal with age-related diseases are focused on treating the symptoms rather than the cause(s). Consequently, elderly populations are taking multiple drugs (9). Decreased drug efficacy, side-effects, and continual deterioration result in a decreased quality of life for our aged population. In conclusion, while the elderly are living longer, they are not living better.

In the heart, many deleterious alterations occur with age that culminates in the deterioration of cardiac function (7). This makes the elderly extremely susceptible to HF and a number of cardiovascular diseases, ultimately leading to a complete loss of pump function. Due to our lack of understanding of the basic mechanisms of aging, there are currently no therapies available to prevent HF in the elder population. It is known, however, that aging is characterized by a persistent low-level oxidative stress, whereby the antioxidant responses no longer stay in balance with oxidant production, leading to damage of lipids, proteins, and
By understanding the mechanisms associated with age-related oxidative stress in the heart, it is possible that we could elucidate the basic biochemical mechanism(s) that initiate aging.

In this dissertation, we sought to elucidate the role of a known pro-inflammatory signaling molecule, ceramide, in cardiac mitochondrial dysfunction. As described in Chapter 1, in vitro studies showed that mitochondrial ceramide accumulation induces a phenotype that resembles the alterations seen in cardiac myocytes with aging. These changes include induction of ROS, inhibition of electron transport, and increased oxidative damage. Interestingly, while much work has been done to understand the possible effects of ceramide accumulation in mitochondria, the mere existence of ceramides in cardiac mitochondria was only inferred and never reported before. In order to determine if ceramide was present in cardiac mitochondria, a LC-tandem mass spectrometry assay was developed to both identify the existence of ceramides, and quantify individual ceramide isoforms. Our results show, for the first time, that cardiac mitochondria contain a small set of ceramides (N-acyl chains of 16 to 24 carbons in length) that contain mostly saturated sidechains. The levels of each individual ceramide had a very low variability, suggesting that their levels are tightly regulated. Furthermore, our results showed that a lipid asymmetry exists, whereby $C_{16}$-, $C_{18}$-, and $C_{24:1}$-ceramide are predominantly found in inner mitochondrial membranes. While the specific role of each ceramide homolog is currently not known, our evidence supports the hypothesis that each species has a specialized role in each membrane.
The existence of ceramides itself does not denote stress nor damage, as they are normal components of lipid membranes. However, *in vitro* evidence shows that even a mild accumulation of ceramide above their normal level results in the induction of stress signaling. Therefore, we sought to determine whether an accumulation of mitochondrial ceramides was phenotypic of the aging heart. Results in chapter three show that, in fact, a ceramide accumulation does occur in cardiac mitochondria with age. Also, the largest accumulations occurred in C\textsubscript{16}-, C\textsubscript{18}-, and C\textsubscript{24:1}-ceramide, the species found primarily within the inner mitochondrial membrane (IMM), suggesting that their most detrimental effect would occur there. This is important because the effects of this ceramide accumulation in the IMM would lead to electron transport inhibition, a commonly known aging phenotype. This evidence supports the theory that aging is akin to low-level inflammation.

Next, we sought to understand why ceramides are accumulating in cardiac mitochondria with age. As described previously, ceramides are primarily formed by two major routes, hydrolysis of sphingomyelin by nSMases and *de novo* synthesis. nSMase activation seemed the plausible route because of the lack of synthetic machinery for *de novo* synthesis in mitochondria. In addition, previous reports have shown that the activity of nSMase is inversely-regulated by physiological concentrations of GSH \((90, 101)\). This is extremely important as there is a marked decrease in mitochondrial glutathione content with age. Our results show that mitochondrial nSMase can be specifically activated by a decrease in mGSH content. These results are important as they tie together, for the
first time, a well-known lesion in antioxidant status to a lipid that induces mitochondrial dysfunction. This also suggests that an effective treatment strategy for age-related mitochondrial dysfunction may lie in the restoration of antioxidant responses, and specifically raising glutathione levels.

In chapter five of this dissertation, we show that treatment with the anti-inflammatory agent LA is able to reverse the age-related ceramidosis. As previously described, LA has been used for many years to treat pro-inflammatory pathologies. It does so, in part, by up-regulating antioxidant response genes, such as those required for glutathione synthesis. The exact mechanism(s) by which LA exerts its effects are still being elucidated. It has been hypothesized that LA induces antioxidant responses by acting as a mild oxidative stressor, with the caveat that it does not, as far as we know, lead to any oxidative damage. This work supports the use of LA as an “age-essential” micronutrient that can combat pro-inflammatory pathologies by increasing the ability of the organism to deal with a stressor. Our findings also support the theory and practice of orthomolecular medicine described by Linus Pauling, and help to expand it further to suggest that we can maintain health and prevent diseases by optimizing nutritional intake; “the right molecules in the right amounts” and also at the correct stage of life.

In conclusion, this dissertation shows that mitochondrial ceramide accumulation is an important phenotype in age-related mitochondrial dysfunction by filling four important gaps in knowledge; 1) ceramides do exist in cardiac mitochondria 2) there is an accumulation of these lipids with age 3) a loss in
mGSH, similar to that seen with age, results in mitochondrial ceramide accumulation in an \textit{in vitro} model, and 4) a two-week feeding of LA is able to restore ceramide levels to that seen in cardiac mitochondria from young animals. As a whole, this dissertation has gathered evidence that supports a new mechanism of cardiac mitochondrial dysfunction in aging and also gives mechanistic insights into the overall health benefits of LA supplementation and supports its use as a safe, natural, “age-essential” micronutrient.

Many questions still exist concerning the nature of ceramides in general as well as in aging and mitochondrial dysfunction. As mentioned throughout this dissertation, it is becoming clear that the N-acyl chain length of ceramides species may be important in their biological function. This is supported by correlative evidence showing that specific chain-lengths accumulate in a variety of disease states (179, 180). Furthermore, in \textit{de novo} synthesis, multiple isoforms of ceramide synthase exist, each responsible for the acylation of a specific sub-set of fatty acid chain lengths (140, 181). This is mostly due to the technical difficulty of modulating the level of a specific chain-length of ceramide. The extreme hydrophobicity of ceramides removes the possibility of accomplishing this by direct incubation procedures as you would a normal cytotoxic agent. Limited success has been achieved using ceramide laden liposomes, but to get them to fuse well requires the use of dodecane (63), which we would expect to have many negative consequences to membrane organization and function.

To this end, we are currently developing a gene-based approach to modulate the levels of specific chain-lengths of ceramide within the mitochondria.
As mentioned previously, there is a family of ceramide synthases (also known as longevity assurance genes, or LASSes) each having an affinity for a different fatty acyl-CoAs as a substrate (e.g. LASS6 preferentially utilizes palmitoyl-CoA and steryl-CoA as a substrate, yielding \(C_{16}\) - and \(C_{18}\)-ceramide, respectively) (140). We have created a plasmid containing LASS6 that has been modified to include the mitochondrial targeting sequence from the pyruvate dehydrogenase E1a subunit (182). By transfecting cells with this vector, we hope target this protein, normally found in the endoplasmic reticulum, to the mitochondria where it can produce \(C_{16}\) - and \(C_{18}\)-ceramide. The core innovation of this project lies in its potential to overcome a serious obstacle in ceramide research by, for the first time, being able to induce a chain-length specific mitochondrial ceramide accumulation. Creation of mitochondrially targeted LASS6, as well as the other LASS isoforms, would allow us to gain the understanding of ceramide function in mitochondria as a whole, and also how individual isoforms of ceramide affect mitochondria.
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Supplemental Figure 1 Asymmetric distribution of cardiac mitochondrial ceramides. Ceramides from IFM and IFM inner membranes were monitored using LC-MS/MS. Ceramides were quantified relative to synthetic internal standards. Mitochondrial samples were normalized based on their phospholipid phosphate content. Data represents the Mean ± SEM, n = 3; an asterisk (*) denotes a significant difference to the corresponding isotype in IMM, p < 0.05.
Supplemental figure 2 Ratio of sphingomyelin to ceramide isoforms.
Sphingomyelin and ceramide isoforms were quantified in mitochondria from freshly isolated hepatocytes using LC-MS/MS. Data is presented as the ratio of sphingomyelin to ceramide.
**Supplemental Figure 3 Acute ceramide accumulation leads to an inhibition in Complex IV activity.** Isolated mitochondria were incubated with a bacterially-derived sphingomyelinase, bSMase, followed by lipid extraction and ceramide quantification by LC-MS/MS. (A) Total ceramide levels were markedly increased by a 20-min bSMase incubation. (B) A time-course of Complex IV activity following incubation with bSMase. Results show a rapid decline in Complex IV activity. Data represent the means ± SEM, n = 3.
Supplemental Table 1 Molecular and product ion transitions used for MRM detection of sphingolipids.