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

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## Abstract

Inflammation results in heightened mitochondrial ceramide levels, which cause electron transport chain dysfunction, elevates reactive oxygen species, and increases apoptosis. As mitochondria in aged hearts also display many of these characteristics, we hypothesized that mitochondrial decay stems partly from an age-related ceramidosis that heretofore has not been recognized for the heart. Intact mitochondria or their purified inner membranes (IMM) were isolated from young (4-6 mo) and old (26-28 mo) rats and analyzed for ceramides by LC-MS/MS. Results showed that ceramide levels increased by 32% with age and three ceramide isoforms, found primarily in the IMM (e.g. $C_{16}$-, $C_{18}$-, and $C_{24:1}$-ceramide), caused this increase. The ceramidosis may stem from enhanced hydrolysis of sphingomyelin, as neutral sphingomyelinase (nSMase) activity doubled 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. Feeding LA [0.2% wt/wt] to old rats for two weeks prior to mitochondrial isolation 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.

### Supplementary key words

electron transport; Complex IV; sphingomyelinase; LC-mass spectrometry



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## 1. 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 [1-8]. 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 [9-11]. 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 [12-20], which increases ROS formation, oxidative stress, and altered energy metabolism upon its accumulation in membranes [21,22].

Generally, acute inflammatory stimuli generate ceramide at the plasma membrane or endoplasmic reticulum by sphingomyelin hydrolysis or *de novo* synthesis, respectively [23-29]. Recent evidence shows that mitochondria may also be an important site of sphingolipid action [22,30-33]. Our laboratory recently showed that cardiac mitochondria normally contain a variety of sphingolipids, including sphingomyelin and ceramide [34]. Mitochondria from other organs also contain ceramide as well as neutral sphingomyelinase (nSMase), which hydrolyzes sphingomyelin to ceramide [35-37]. 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 [21,22,31-33]. 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. Lastly, if mitochondrial ceramides were indeed found to become elevated with age, a contingent goal was to determine whether anti-inflammatory agents could remediate any ceramide accumulation, thereby ameliorating the mitochondrial aging phenotype.

With regard to this latter contingent goal, our laboratory and others showed that the dithiol compound, (*R*)-α-lipoic acid (LA) may act as a potent anti-inflammatory and anti-oxidant agent at pharmacological doses [38-42]. 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 [43]. We have also previously shown that LA lowers indices of mitochondrial dysfunction [43-45], thus providing a rationale that LA may reverse at least certain aspects of mitochondrial decay by opposing ceramidosis.

## 2. Materials and Methods

### 2.1 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.

### 2.2 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 in Weniger Hall, Oregon State University, and maintained by the Department of Laboratory Animal Resources and Care. All animal procedures were performed in accordance with the Oregon State University guidelines for animal experimentation.

### 2.3 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 had 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 diethyl ether 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.

### 2.4 Mitochondrial isolation

Cardiac mitochondria were isolated using differential centrifugation as described by Palmer *et al.* [46] with modifications as in Monette *et al.* [34]. This procedure resulted in an enriched interfibrillary 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).

### 2.5 Inner mitochondrial membrane (IMM) isolation

The outer mitochondrial membrane (OMM) was selectively removed using digitonin [47]. 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 $KH_2PO_4$, 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.

### 2.6 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*.

### 2.7 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 Nikolova-Karakashian [48].

### 2.8 Measurement of glutathione (GSH)

GSH was conjugated to dansyl chloride and measured by using HPLC and fluorescence detection as described by Dixon *et al.* [49].

### 2.9 Lipid extraction

All samples were prepared as in Merrill *et al.* [50] except that samples were not saponified in KOH.

### 2.10 LC-tandem mass spectrometry

Lipids were separated by HPLC using a Supelco Discovery column (2 mm × 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.* [34]. Quantitation was based on comparison to synthetic sphingolipid standards.

### 2.11 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. Results

### 3.1 Profile of cardiac mitochondrial sphingolipids

In keeping with our previous work [34], 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 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.

### 3.2 Age-related mitochondrial ceramide accumulation

On an age basis, total mitochondrial ceramide increased by 32% (Figure 2). Analysis of individual isoforms showed that the age-related elevation in ceramide stemmed from increases in all ceramide species (Table I). For 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 I). 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 section 3.1) are the ones that increased the most with age. This suggests that the age-related ceramide accumulation primarily occurs in the IMM.

### 3.3 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 2). Furthermore, only modest changes in ceramide isoforms were noted when comparing young LA-treated animals to the age-matched controls (Table I). 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 2). 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 I). 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 I). 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.

### 3.4 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 [21,22], 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), which is in keeping with previous literature reports [3,51]. 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 (Supplemental Figure 1A). Elevation of ceramide levels via bSMase resulted in a marked 62% loss in Complex IV activity versus controls (Supplemental Figure 1B). 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.

### 3.5 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 3). This indicates that LA does not directly modify cytochrome oxidase *per se*. However, for old rats, LA reversed the loss of Complex IV function where its activity was no longer different from young animals (Figure 3). We therefore conclude that LA modulates Complex IV activity only in mitochondria from aged tissue where elevated IMM ceramides are evident.

### 3.6 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 sphingomyelinases, 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 4); 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.

Because mitochondria reportedly also contain ceramidases, which catabolize ceramide to sphingomyelin [52], 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.

### 3.7 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 [27,53,54] and GSH levels decline markedly in the aging heart, we hypothesized that the LA-mediated improvement in mitochondrial ceramide status was through its means for restoring mitochondrial GSH levels [2,20]. As anticipated, LA-treatment did not alter GSH levels in young versus controls. However, LA reversed the 43% age-associated decline in mitochondrial GSH levels, such that the loss was no longer statistically different than in young untreated rats (Figure 5). 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.

## 4. 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 [55], myocardial infarct [56,57], Type II diabetes [58], as well as *in vitro* experiments where isolated mitochondria were treated with ceramide-laden liposomes [21,22], 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 [59]. Also, Straczkowski *et al.* reported that men at risk for diabetes have a 50 to 200% increase in type II muscle ceramide levels [15]. 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 highest with age (Table 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 $C_{18}$-ceramide, but not by orthologs containing even one site of unsaturation [60]. $C_{16}$- and $C_{18}$-ceramide appear to be pro-apoptogenic ceramides and commonly increase during pathological conditions [60-62]. 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 mainly 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.

Another 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 from young rats. Although the pharmacological mechanism by which LA reverses the evident ceramidosis and ETC dysfunction solely in old rats is not fully understood, our data suggest that LA works by limiting age-dependent increases in mitochondrial nSMase activity. This most likely occurs through LA-dependent restoration of GSH levels, as there is an inverse correlation between nSMase activity and GSH, where low GSH status activates the enzyme by increasing its Vmax [53,54]. We previously showed that the GSH redox status of the myocardium and cardiac interfibrillary mitochondria is altered with age, and that LA treatment corrects these changes [63-67]. 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.

Finally, 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 [68]. 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 [19]. Thus, LA may be an appropriate adjunct to limit age-related mitochondrial ceramidosis and the adverse cardiac effects that its accumulation causes.

## 5. Conclusions

In conclusion, this paper shows that the age-related decay in cardiac mitochondria may stem from an accumulation of ceramide, a pro-apoptotic signaling lipid shown to induce mitochondrial dysfunction. Furthermore, we show that a two-week feeding of LA is able to restore ceramide levels to that seen in cardiac mitochondria from young animals. These results thus highlight the role of LA as an age-essential micronutrient, which may be an effective adjunct in preventing or reversing mitochondrial-associated pathologies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **LA** | (*R*)-alpha-lipoic acid |
| **GSH** | glutathione |
| **LC-MS/MS** | LC-tandem mass spectrometry |
| **ETC** | electron transport chain |
| **nSMase** | neutral sphingomyelinase |
| **bSMase** | bacterial sphingomyelinase |
| **ER** | endoplasmic reticulum |
| **OMM** | outer mitochondrial membrane |
| **ROS** | reactive oxygen species |
| **IMM** | inner mitochondrial membrane |

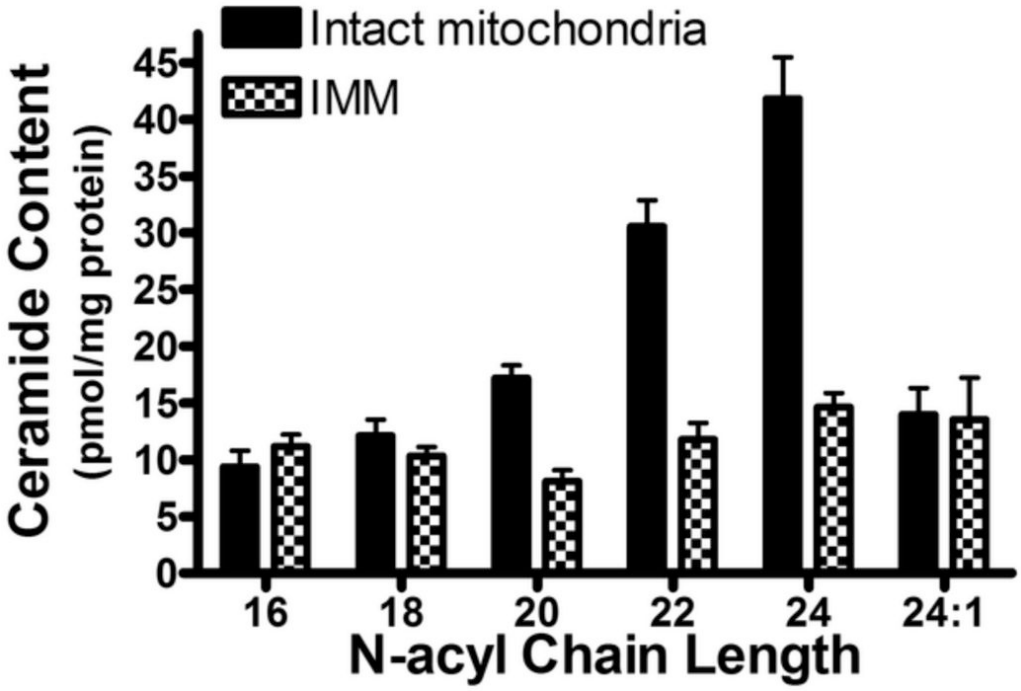


**Figure 1. Asymmetric distribution of cardiac mitochondrial ceramides**

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.

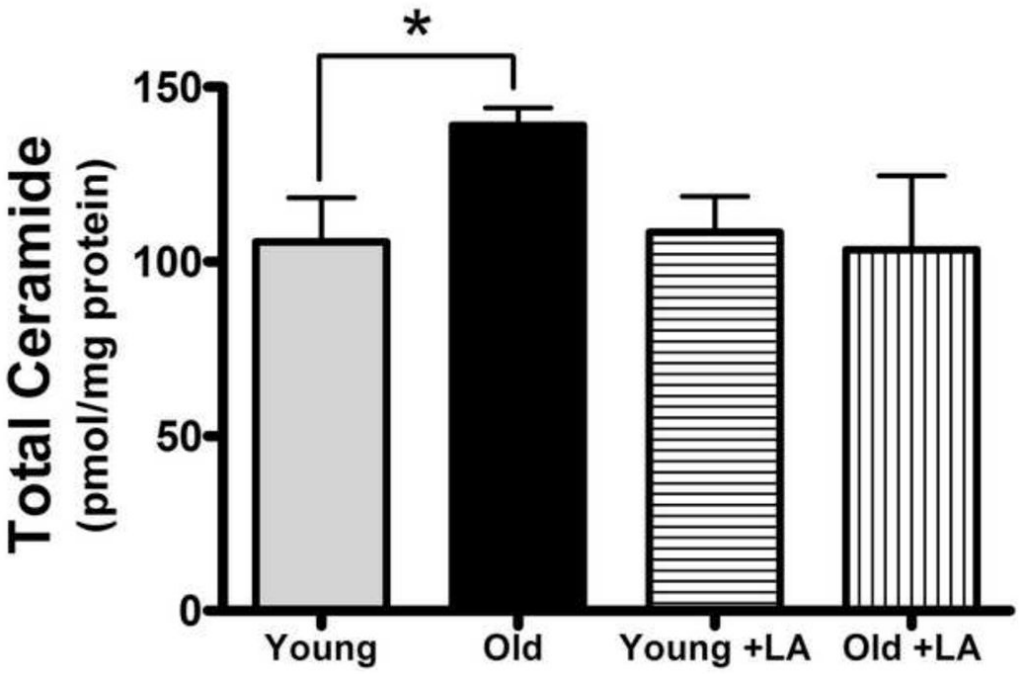


**Figure 2. LA treatment decreases mitochondrial ceramides**

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, $p < 0.03$.

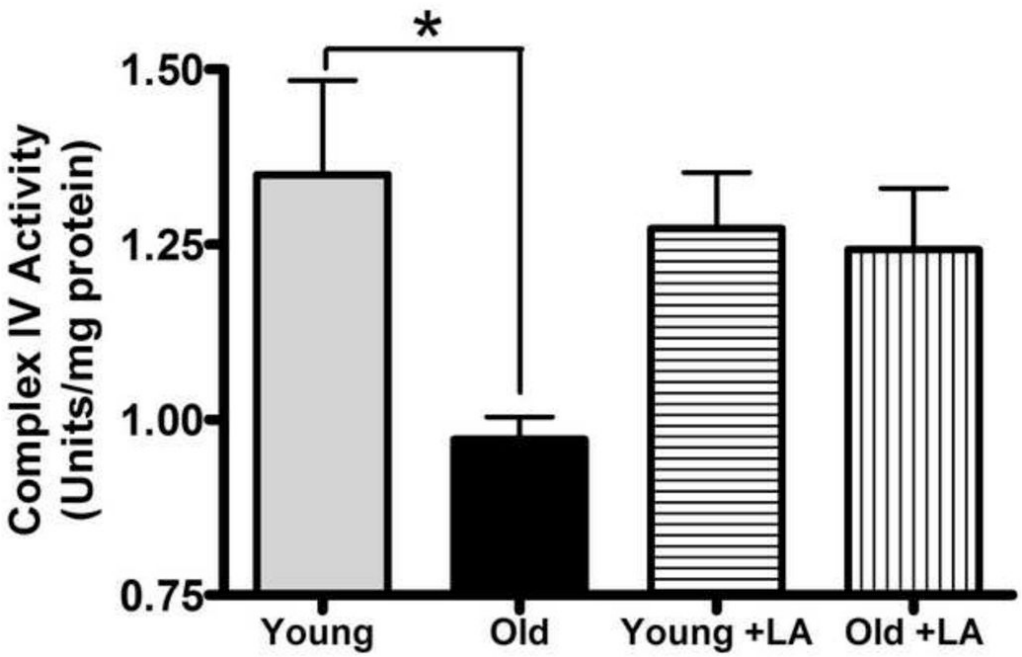


**Figure 3. LA treatment restores Complex IV activity in cardiac mitochondria from aged animals**

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, $p \leq 0.05$.

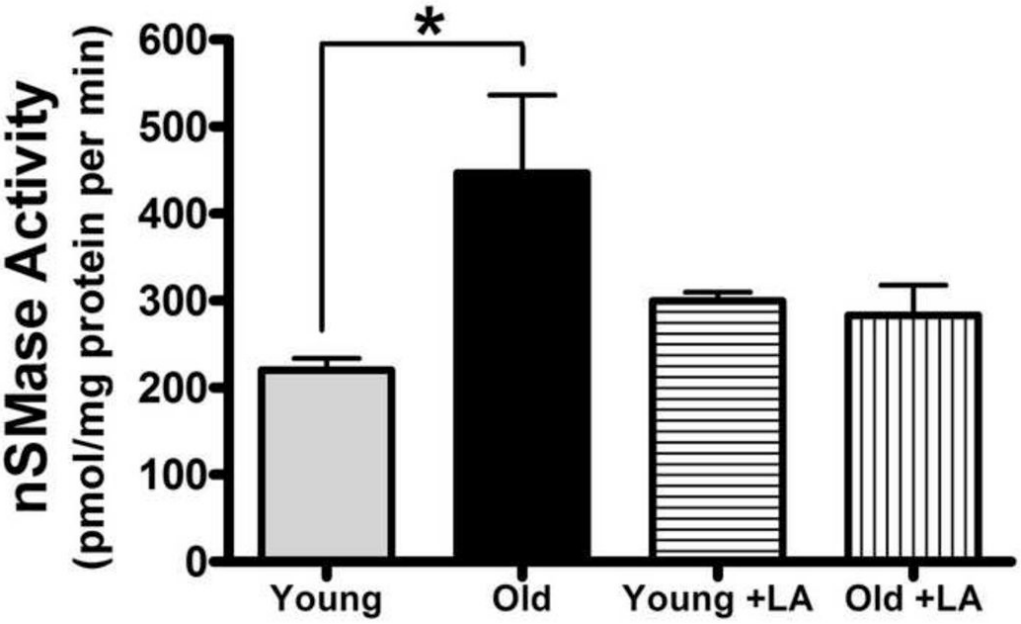


**Figure 4. LA restores neutral sphingomyelinase (nSMase) activity in mitochondria from old animals to youthful levels**

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, $p < 0.01$.

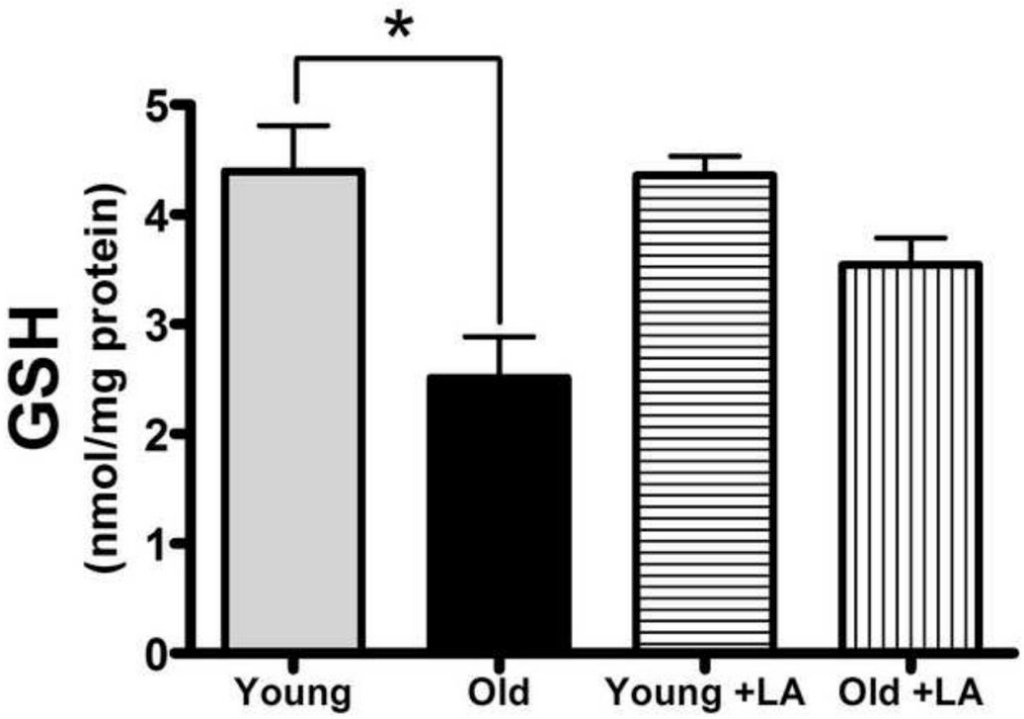


**Figure 5. LA markedly increases mitochondrial glutathione levels that otherwise decrease with age**

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, $p \leq 0.05$.

**Table I**

**Cardiac mitochondrial ceramide levels with and without lipoic acid supplementation**

| Ceramide Species | Young Control | Old Control | | Young +LA | | Old +LA | | |
|---|---|---|---|---|---|---|---|---|
| | pmol/mg protein | pmol/mg protein | Δ% from Young Control | pmol/mg protein | Δ% from Young Control | pmol/mg protein | Δ% from Young Control | Δ% from Old Control |
| $C_{16}$-ceramide | 7.4 ± 0.6 | 12.7 ± 1.9* | 72.3 | 8.0 ± 0.6 | 8.2 | 8.7 ±2.1 | 18.3 | −31.3 |
| $C_{18}$-ceramide | 7.8 ± 1.3 | 13.5 ± 1.2* | 73.4 | 8.2 ± 1.0 | 4.7 | 8.7 ± 1.5# | 11.4 | −35.7 |
| $C_{20}$-ceramide | 12.5 ± 1.5 | 14.8 ± 1.1 | 18.1 | 13.2 ± 2.2 | 5.8 | 10.3 ± 1.9 | −17.5 | −30.1 |
| $C_{22}$-ceramide | 27.7 ± 2.6 | 30.0 ± 0.9 | 8.0 | 31.7 ± 2.7 | 14.2 | 25.6 ±6.1 | −7.6 | −14.4 |
| $C_{24}$-ceramide | 40.6 ± 6.4 | 48.8 ± 5.6 | 20.0 | 38.2 ± 3.1 | −6.0 | 35.9 ± 7.4 | −11.6 | −26.4 |
| $C_{24:1}$-ceramide | 10.9 ± 1.8 | 19.4 ± 4.2 | 77.7 | 9.3 ± 1.4 | −15.1 | 14.2 ± 2.5 | 30.2 | −26.7 |

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

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