AN ABSTRACT OF THE DISSERTATION OF

Luis A. Gómez for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on December 07, 2012.

Title: Mitochondrial Decay in the Aging Rat Heart: Changes in Fatty Acid-Supported Bioenergetics and Macromolecular Organization of the Electron Transport System.

Abstract approved:

Tory M. Hagen

Decline in cardiac pump function is a hallmark of aging where mitochondrial decay is an important underlying cause. Although certainly multifactorial in nature, both dysfunction of the machinery involved in the chemiosmotic process of energy transduction and lower capacity to maintain fatty acid-driven respiration are identified as intrinsic factors of mitochondrial decay in the aged myocardium.

Age-associated destabilization of electron transport supercomplexes as a potential factor of mitochondrial decay in the rat heart. Defective operation of the electron transport chain (ETC) constitutes a key mechanism involved in the age-associated loss of mitochondrial energy metabolism.

Nevertheless, the molecular events underlying inefficient electron flux that ultimately leads to higher superoxide appearance and impaired respiration are not fully known. As recent biophysical evidence shows that the ETC may form large macromolecular assemblies (i.e. supercomplexes) that disintegrate in certain pathologies (e.g. heart failure or Barth syndrome) reminiscent of aging, we investigated the hypothesis that alterations in supercomplexes are partly responsible for the age-related loss of cardiac ETC function.

In this dissertation, age-associated changes in supercomplex organization and stability were investigated in subsarcolemmal (SSM) and interfibrillary (IFM) mitochondria isolated from cardiac tissue from young (3-5 months) and old (24-28 months) male Fischer 344 rats.

Blue native-PAGE (BN-PAGE) analysis of digitonin-solubilized mitochondrial membranes coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to investigate supercomplex organization. Results show that both SSM and IFM display supercomplexes comprised of various stoichiometries of complexes I, III and IV (never complex II), which typically organize as high mass (1500-2300 kDa) assemblies containing up to four copies of complex IV (i.e. $I_1III_2IV_N$-type supercomplexes). Interestingly, analysis of IFM proteins showed that, in

general, supercomplex levels declined by up to 15 % ($p < 0.05$) with age; however, different degrees of supercomplex deterioration were observed, depending on the particular supercomplex investigated. Supercomplexes of the highest molecular weights (i.e. 1900-2300 kDa), which were also composed of the most complex stoichiometries (i.e. $I_1III_2IV_N$, $N \geq 2$), were primarily lost with age. In particular, $I_1III_2IV_2$, $I_1III_2IV_3$ and $I_1III_2IV_4$ supercomplexes were found to decline by 13% ($p < 0.05$), 30% ($p < 0.05$) and 45% ($p < 0.05$), respectively, on an age basis. Therefore, the age-associated loss of supercomplexes in IFM stems from destabilization of the assemblies that comprise several copies of complex IV, which could partially limit proper electron transfer to O_2 for its reduction, affecting mitochondrial respiratory capacity.

In contrast to IFM, the aging defects of SSM supercomplexes appeared to be confined to the assembly comprised of only one copy of complex IV ($I_1III_2IV_1$, 1700 kDa) (37% loss; $p = 0.06$), while the higher molecular weight supercomplex sub-types that were most affected in IFM (i.e. $I_1III_2IV_N$, $N \geq 2$) were not significantly altered with age. Thus, the results from this dissertation indicate that mitochondria from different subcellular locations in the myocyte show different degrees of supercomplex destabilization in the aging rat heart. The more robust

supercomplex deficits noted for IFM fit well with previous observations that electron transport characteristics of this subpopulation are more adversely affected with age than SSM.

Although the underlying factor(s) of supercomplex deterioration are not fully known, the hypothesis that age-related alterations of certain constituents of the IMM (e.g. cardiolipin) may be important factors of supercomplex destabilization in cardiac mitochondria was investigated in this dissertation. To this end, LC-MS/MS characterization of supercomplex proteins and HPLC analysis of cardiolipin were used as approaches to elucidate potential factor(s) of supercomplex destabilization in the aging rat heart. Age-related alterations of cardiolipin levels and its acyl-chain content showed a strong parallel to the age-associated destabilization of supercomplexes. Specifically, cardiolipin levels declined by 10% ($p < 0.05$) in IFM, the mitochondrial subpopulation displaying the highest degree of supercomplex deterioration. In addition, the content of $(18:2)_4$-cardiolipin, the predominant species in the heart, was found to decline by 50% ($p < 0.05$) on average in both populations of cardiac mitochondria. Therefore, the data presented in this dissertation indicate that changes in cardiolipin may be at least one of the factors involved in supercomplex destabilization in the aging heart.

Age-related decline in carnitine palmitoyltransferase I (CPT1) activity as a mitochondrial lesion that limits fatty acid catabolism in the rat heart. Loss of fatty acid utilization, another intrinsic factor of mitochondrial decay in the aged myocardium, has been associated with age-related alterations in the activity of carnitine palmitoyltransferase 1 (CPT1), the rate-controlling enzyme for overall fatty acid β-oxidation. Nevertheless, the exact molecular mechanism involved in the age-related loss of fatty acid-driven bioenergetics is not fully understood. In this dissertation, it was also investigated whether the aging lesion for fatty oxidation lies in a particular mitochondrial subpopulation or more generally results from cardiac decrements in L-carnitine levels. In order to clarify the role of each one of these factors, the effect of long-term dietary supplementation with the L-carnitine analogue, acetyl-L-carnitine (ALCAR), was also investigated.

Results show that aging selectively decreases CPT1 activity in IFM by reducing enzyme catalytic efficiency for palmitoyl-CoA. IFM displayed a 28% ($p < 0.05$) loss of CPT1 activity, which correlated with a decline (41%, $p < 0.05$) in palmitoyl-CoA-driven state 3 respiration. Interestingly, SSM had preserved enzyme function and efficiently utilized palmitate. Analysis of IFM CPT1 kinetics showed both diminished V_{max} and K_m (60% and 49% respectively, $p < 0.05$) when palmitoyl-CoA was the substrate.

However, no age-related changes in enzyme kinetics were evident with respect to L-carnitine. ALCAR supplementation restored CPT1 activity in heart IFM, but not apparently through remediation of L-carnitine levels. Rather, ALCAR influenced enzyme activity over time, potentially by modulating conditions in the aging heart that ultimately affect palmitoyl-CoA binding and CPT1 kinetics.

In conclusion, this dissertation presents a characterization of age-associated alterations in the macromolecular organization of the IMM components that could partly explain the loss of mitochondrial oxidative capacity that affects the aging heart. In addition, the characterization of an age-related lesion of the controlling enzyme for β-oxidation is presented as another important factor that limits mitochondrial function and energy metabolism in cardiac mitochondria.

Mitochondrial Decay in the Aging Rat Heart: Changes in Fatty Acid-Supported Bioenergetics and Macromolecular Organization of the Electron Transport System

by

Luis A. Gómez

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented December 07, 2012

Commencement June 2013

Doctor of Philosophy dissertation of Luis A. Gómez presented on December 07, 2012.

APPROVED:

Major Professor, representing Biochemistry and Biophysics

Head of the Department of Biochemistry and Biophysics

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.

Luis A. Gómez, Author

ACKNOWLEDGEMENTS

For all You have permitted,
Thank You God.

I want to take this opportunity to thank my Major Professor, Dr. Tory M. Hagen, for his enormous effort and dedication to get me to succeed as a graduate student and a Ph.D. candidate. From the deepest of my heart, I want to thank him for believing in me and also for giving me the opportunity of pursuing a dream. I feel truly fortunate that I could work under the guidance of such a great scientist, at the time that he gave me his friendship through these years of my life as a student at Oregon State University. All I have to say is that being part of the Hagen lab really made a difference in my life.

I also want to thank the members of my research committee, Drs. Claudia S. Maier, Balz Frei, Joseph Beckman and Adam Higgins not only for their comments and feedback about my research project but also for their help and collaboration, which went beyond their official role as members of my thesis committee.

I am truly thankful with all the members of the Hagen lab, a crowd that was a second family to me and always made feel like I was at home,

even though I was thousands of miles away from my native home. All my gratitude to Judy A. Butler and Shi-Hua Du Heath for making my life sweet and wonderful, and for taking care of me through all these years. They both will always have a special place in my heart. I also want to thank former members of the Hagen lab, Brian Dixon and Jeffrey Monette, who guided me during my first years as a graduate student by sharing all their experience as preceding students. My gratitude also goes to Kate Shay, Keith Dove, Régis Moreau, Eric Smith, Liam Finlay and Nicholas Thomas, who every day tried to make my life in the lab much easier. Needless to say, their help was a very important contribution to my success.

I also want to extend my gratitude to the Department of Biochemistry and Biophysics as well as to the Linus Pauling Institute for providing me with a wonderful education that has made an enormous impact on my life.

Finally, I want to thank my family for all their love and support all these years. They are truly the best gift from God.

CONTRIBUTION OF AUTHORS

I want to acknowledge Dr. Jeffrey S. Monette because his ideas were very important to develop the experimental approach that was used in Chapter 2. I also want to thank his significant help in performing the mitochondrial isolation work that was necessary for the experiments presented in this chapter.

Also, I would like to acknowledge the contributions of Drs. Claudia S. Maier and Juan D. Chavez who developed the mass spectrometry-based protocol that was used in Chapter 2. The proteomic analysis they performed of mitochondrial proteins was very important for the characterization of supercomplexes presented in this chapter.

Mrs. Shi-Hua D. Heath must be acknowledged for her contribution in implementing the experimental protocols and performing most of the experimental work that is presented in Chapter 5.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

To my parents

For all their love and support

Chapter 1

General Introduction

Some sections of this chapter are excerpts from the following review article:

Age-related decline in mitochondrial bioenergetics: Does supercomplex destabilization determine lower oxidative capacity and higher superoxide production?

Luis A. Gómez and Tory M. Hagen

Seminars in Cell and Developmental Biology
23 (2012) 758– 767

Elsevier Inc. (Corporate Office)
360 Park Avenue South,
New York, NY 10010

1.1 General Background and Significance

1.1.1 Cardiac function in the aging process

Aging limits cardiac reserve capacity [1, 2], which increases deficits of daily living and augments the risk for heart failure (HF) [3-5], the leading cause of hospitalization among the population over the age of 65 [6-8]. Alterations of myocardial energy metabolism and changes in the structure of the contractile apparatus of the heart are identified as underlying factors of cardiac dysfunction in the aging animal [9-12]. Age-associated fibrosis and increased apoptosis have been characterized as main factors of the ventricular remodeling process that results in diastolic dysfunction in humans [13, 14] and rodents [9, 11]. For instance, in a study where heart specimens were analyzed in subjects ranging from 19 to 104 years of age, Kajstura et al. showed that the population of apoptotic myocytes increases with age [15]. According to these authors, because cell death via apoptosis overwhelms the generation of new cells, the population of post-mitotic myocytes significantly declines with age in humans [15]. Thus, even though the mammalian heart is a self-renewing organ, it has a limited capacity of myocyte replacement [15], which appears to contribute to the age-related accumulation of atrophied ventricular tissue [9-12].

Also, using ^{31}P magnetic resonance (MR) coupled with chemical shift imaging (CSI), Schocke et al. showed that aging decreases the phosphocreatine (PCr)-to-ATP ratio in human subjects [12], which correlated with development of diastolic dysfunction and hypertrophy of the left ventricle [12]. Therefore, a parallel exists between alterations in cellular ATP synthesis and turnover, and the myocyte loss that adversely affects the aging heart.

The molecular mechanism(s) that mediate myocyte apoptosis and impaired energy metabolism in the aging heart are not fully known; however, this organ has a highly aerobic nature and mitochondria constitute around 35% of the myocardial cell volume [16]. Thus, because mitochondria play a key role not only in maintaining cardiac aerobic metabolism and energy supply [17, 18] but also in mediating both Ca^{2+} homeostasis and cytochrome *c* release and apoptosis initiation [19-21], there is a rationale that deterioration of mitochondrial function adversely affects cardiac reserve in the aging animal. For instance, using gene profiling studies in C57BL/6 mouse myocytes, Bodyak et al. reported a decrease in the transcript levels of mitochondrially encoded subunits of complexes I, III and IV [22]. Moreover, the findings reported by Bodyak et al. parallel several reports of age-related alterations in both structure and

activity of complexes I, III and IV of the electron transport chain (ETC) in cardiac mitochondria [23-26]. Therefore, understanding the biochemical factor(s) that cause mitochondrial dysfunction and impair aerobic energy metabolism in the aging heart is key to developing strategies aimed to slow down the progression of changes that limit cardiac reserve with age. This dissertation mainly focuses on investigating certain molecular mechanisms that may contribute to mitochondrial decay in the aging myocardium. In particular, dysfunction of the ETC machinery and alterations in fatty acid-driven respiration were investigated in the aging rat heart.

1.1.2 Mitochondrial dysfunction in the aging process

General aspects regarding the etiology of mitochondrial decay in aging

Mitochondrial decay plays a central role in the aging process [27]. Although the age-associated loss of mitochondrial function is undoubtedly multifactorial, several lines of evidence indicate that certain molecular and cellular alterations are significantly involved in the progression of changes that ultimately lead to impaired mitochondrial energy metabolism. Age-related oxidative damage and deletions of mitochondrial DNA (mtDNA) that correlate with lower respiratory activity have been reported in liver [28]

and cardiac muscle [29] of rats, liver [30] and skeletal muscle [31] of primates, and skeletal [32] and cardiac muscle [29, 33] of humans. Moreover, mtDNA deletions accumulate during the progression of atrial fibrillation in human aging [33]. On the other hand, impaired autophagy that regulates mitochondrial homeostasis also appears to contribute to the age-related accumulation of respiratory defects in tissues [34-36]. In this regard, Terman et al. showed that inhibition of autophagy in neonatal rat cardiomyocytes leads to morphological alterations of mitochondria and changes in membrane potential ($\Delta\psi_m$), similar to that of senescent cells [37]. This group further concluded that abnormal mitochondria accumulate in cardiac tissue as a consequence of a defective autophagy process [37, 38].

It has been hypothesized that the aforementioned characteristics of mitochondrial decay are symptomatic of a vicious downward spiral where defective ETC complexes contribute to enhanced reactive oxygen species (ROS) generation by mitochondria, which in turn increases mtDNA damage and mutations, which eventually reciprocally affects structure of the ETC components (for recent reviews see [39, 40]). Ultimately, higher rates of ROS appearance increase oxidative modification of membrane lipids and proteins to the point where accumulation of molecular defects overcomes

the capacity to maintain mitochondrial homeostasis through autophagy [34, 37, 40].

Consequences of mitochondrial decay on cellular function

An immediate consequence of the age-related impairment of mitochondrial function would be decreased aerobic energy transduction. A consistent picture has emerged from examination of mitochondrial bioenergetics in intact cells from young and old animals of many species. Here, mitochondrial membrane potential ($\Delta\psi_m$) and cellular energy status progressively decline with age in both freshly isolated rat hepatocytes [28] and in human skin fibroblasts taken from very young (fetal material) versus very old (103 years) human donors [41]. This work is now buttressed by reports connecting mitochondrial decay to organ decline *in vivo*. Using ^{31}P magnetic resonance spectroscopy and optical approaches to simultaneously monitor ATP synthesis and O_2 uptake, Marcinek et al. showed that working skeletal muscle of aged C57BL/6 mice experienced a 50% decline in the mitochondrial P/O ratio, an indicator of the efficiency of ATP synthesis coupled to respiration, and a consequent loss in energy charge versus young animals [42]. This is in agreement with Kostler et al. who observed that high-energy phosphates in the human heart declined significantly *in vivo*

with age, suggesting energy reserve capacity in this organ is highly attenuated [43]. Furthermore, using positron emission tomography for analyzing cardiac function in humans, Kates et al. found that aging significantly decreases fatty acid oxidation rates, with respect to myocardial oxygen consumption rates [44].

In addition, aging reportedly diminishes State 3 respiration rates during oxidation of NADH-associated substrates in rat mitochondria isolated from hippocampus and brain cortex [45, 46], skeletal muscle [47], liver [48], kidney [49], and heart [23, 25, 47, 50], as well as in human skeletal muscle mitochondria [51]. Nevertheless, it is less clear whether aging modifies respiratory control (RCR) and P/O ratios when using *in vitro* models. Experiments on isolated rat heart mitochondria showed that even though State 3 respiration declines with age, the P/O ratio and RCR remain unaffected [25, 47, 52]. In line with these findings, O'Toole et al. reported a lack of age-related changes in the P/O and RCR in rat kidney mitochondria [49]. In contrast, other reports reveal that RCR values decline with age in rat liver mitochondria [48] and also in mitochondria from rat hippocampus [46]. To summarize, aging decreases mitochondrial ADP-stimulated respiration and cellular aerobic metabolism, but the degree of this decline is

controversial. *In vitro* studies may not precisely reflect the magnitude that age-related mitochondrial decay impairs cellular energy metabolism *in vivo*.

Analysis of mitochondrial calcium handling, a key aspect of overall mitochondrial function, shows that aging induces higher vulnerability to calcium overload and propensity to permeability transition in mitochondria in brains and livers from B6D2F1 mice [53] and in interfibrillar mitochondria from Fischer 344 [50] and Fischer 344 x Brown Norway rats [54]. Furthermore, Hofer et al. showed that the age-related loss of calcium-accumulating capacity in mitochondria from Fischer 344 x Brown Norway rats correlated with higher concentrations of cytosolic cytochrome *c* and resultant higher caspase 3 activity [54]. In further support for these observations, it was recently observed that human cardiomyocytes display higher expression of proapoptotic proteins (e.g. Bax) with age, which correlate with increased cytosolic levels of cytochrome *c* and caspase 9 [55].

Lastly, a strong relationship has emerged between mitochondrial decay and oxidative damage to mitochondrial and extramitochondrial cellular components (for reviews see [39, 40, 56, 57]). Consistent with this view are studies, showing higher levels of mtDNA deletions and oxidative damage (see above), oxidative DNA damage [55, 58], lipid peroxidation

[58-61], and lipid adduction to proteins [62-64] with age. Cardiolipin, a mitochondrial specific phospholipid, may be particularly prone to oxidative damage and loss (see Section 1.1.3 and also [65]). This is because of the highly unsaturated nature of its acyl side-chains and its proximity to ROS emanating from the ETC [66-69]. In this respect, a characterization of age-related alterations in cardiolipin from rat heart mitochondria is presented in Chapter 4 (see also Section 1.1.3).

Additionally, most amino acid residues can be oxidized by ROS, which lead to formation of disulfide bonds and carbonyl derivatives [55, 70-74]. Proteins in proximity to decaying mitochondria may be particularly susceptible to oxidative damage and dysfunction. Thus, mitochondrial-driven oxidative damage may not only influence progression of mitochondrial decay but also adversely affect cell survival and overall organ function.

Even though the aforementioned characteristics of mitochondria in aged tissues show varying degrees of alterations, the available evidence nevertheless supports the view that that electron flux efficiency through the ETC declines while oxidant leak is enhanced with age. Both of these age-associated deficits emanate from an altered inner mitochondrial membrane (IMM). The following section will provide evidence for the functional

consequences of mitochondrial decay, especially with respect to ETC function.

1.1.3 Age-associated changes of the mitochondrial energy transduction system

Catalytic and structural alterations of the respiratory chain complexes in aging

Dysfunction of the components of the ETC strongly correlates to the age-related mitochondrial decline in bioenergetic reserve observed in different organs [23-25, 75]. In both rodents and humans, complex I catalytic activity declines with age in liver [24, 76], brain [46, 77], and heart [24, 55, 78, 79]. In addition, several alterations of the complex III holo-protein result in its lower activity, particularly in post-mitotic tissues of aging rodents [25, 75, 78, 80] and primates [81]. These results agree with other studies showing that defects of complex IV correlate with lower mitochondrial oxidative capacity in post-mitotic tissues of elder humans [82, 83], primates [81, 84], and rodents [23, 26, 46, 85-87].

Age-associated alterations of the ETC components promote inefficient electron transport (i.e. higher electron leakage) and increased

ROS appearance in mitochondria [79, 80, 88, 89]. In turn, higher ROS generation contributes to ETC dysfunction by initiating oxidative modifications of ETC proteins [26, 63, 78] and mtDNA [31, 90] (see also Section 1.1.2). However, the mechanism(s) of superoxide ($O_2^{\bullet-}$) generation under physiological circumstances are not completely understood, and the role that the ETC plays in increased $O_2^{\bullet-}$ with age is not yet clear. In this regard, experimental observations by Moghaddas et al. indicate that oxidative modification of the Q_o binding site in complex III is the main factor involved in higher $O_2^{\bullet-}$ generation in the aged heart [80]. On the other hand, complex I may also be an important source of $O_2^{\bullet-}$ generation that could contribute to enhanced $O_2^{\bullet-}$ with age [79, 89, 91, 92].

Regardless of the specific site of production, increased ROS from an impaired ETC correlates with a decline in mitochondrial antioxidant status with age. The mono-thiol antioxidant, glutathione (GSH), is particularly diminished in mitochondria from aged tissue. It has been shown that aging leads to deficits in both mitochondrial GSH levels and its redox ratio (GSH/GSSG) in the brain and heart of rats [93]. Moreover, we observed both an age-related loss of GSH and ascorbate in rat hepatocytes [94] and isolated rat heart mitochondria [26]. The aging rat heart also displayed significantly lower ascorbate concentrations [58]. Thus, mitochondria from

aged tissues of a variety of species display increased ROS output, lower antioxidant defenses, and greater oxidative damage.

Age-related changes in the lipid composition of the inner membrane

Aging leads to alterations of the lipid composition of the IMM that also contribute to impaired bioenergetic capacity of organs and tissues. An age-related decline in coenzyme Q, a key isoprene-derivative that mediates electron transfer between several IMM protein complexes, has been observed in plasma [95] and also in cardiac muscle from human subjects [96, 97]. In addition, tissue levels of coenzyme Q appear to decline in heart, kidney, and skeletal muscle from aged rats [98]. This loss appears to be more pronounced when coenzyme Q levels are measured in isolated mitochondria versus whole tissues [99, 100]. Additionally, multiple studies suggest that aging leads to lower cardiolipin levels and/or its acyl side-chain composition. Cardiolipin is a phospholipid that is almost exclusively located to mitochondria and acts as an important cofactor of several IMM proteins [66, 67]. Studies using brain [77], liver [101], and cardiac mitochondria [102-107] as well as isolated rat hepatocytes [94, 108] indicate that cardiolipin significantly declines with age. However, the extent of cardiolipin loss and/or whether such declines are functionally

consequential is controversial. Hoppel and colleagues provided evidence that interfibrillar mitochondria of the aging rat heart have no changes in cardiolipin levels or composition, in contrast to earlier reports [109]. The reason for the discrepancy between this work and the aforementioned studies is not clear, although a partial answer may lie in the different methods used to extract lipids from mitochondrial fractions (e.g. one-phase organic systems versus two-phase mixtures) and the various techniques employed for separation and analysis of cardiolipin.

Despite the controversial degree of general cardiolipin loss, there is a growing consensus that significant remodeling of cardiolipin acyl side-chains occurs with age. Helmy et al. observed an age-related increase in the ratio of monolyso-cardiolipin to mature cardiolipin in the guinea pig kidney [110], an indicator of defective incorporation of acyl chain units into cardiolipin during the remodeling cycle [111]. In addition, tetralinoleoyl-cardiolipin ($[18:2]_4$-cardiolipin), the predominant species in the heart [69, 112], appears to be the most adversely affected on an age basis [113]. Results presented in Chapter 4 indicate that the content of $(18:2)_4$-cardiolipin declined by 50% on average in two different populations of cardiac mitochondria; however, only a modest (~10% on average) loss in global cardiolipin levels was found in interfibrillar mitochondria from old

animals, relative to young controls. These results partially agree with the observations made by Hoppel and coworkers [109]. Alteration of acyl side-chains, as evident in aging tissues, would be expected to adversely affect ETC electron movement because of protein conformational changes. Thus, cardiolipin molecular composition is markedly affected with age, possibly due to defects in remodeling of mature cardiolipin.

Although aging does not significantly affect the levels of major phospholipids (e.g. phosphatidylethanolamine) [101, 104, 106, 107], the overall IMM composition and its fluidity are significantly changed with age. Cholesterol accumulates in the IMM, alters membrane fluidity, and may promote greater H^+ leakage [101, 106]. In a similar vein, using LC-MS/MS analysis, we recently demonstrated that aged cardiac mitochondria accumulate ceramide, a pro-apoptotic sphingolipid, which results in inhibition of complex IV activity [87]. Furthermore, three ceramide isoforms in the IMM (e.g. those with 16, 18, and 24:1 acyl chains) caused the increase in overall ceramide levels [87]. Thus, extensive age-related alterations of the lipid milieu of the IMM occur, which adversely affect membrane structural organization and contribute to limiting the biological function of the ETC complexes.

1.1.4 Supercomplex destabilization as a new underlying factor of mitochondrial decay in aging

Supercomplex organization of the electron transport chain

The ETC comprises four large protein complexes (Fig. 1.1), which along with the F_1F_O-ATP synthase (complex V) and mobile electron carriers (e.g. cytochrome *c* and coenzyme Q), constitute the machinery for converting metabolic energy transiently stored as reduced coenzymes into ATP. Until recently, the prevailing view was that the components of the ETC were distinct entities where rapid, random collisions allowed electron transfer between complexes (Fig. 1.1A) [114-117]. However, with the advent of Blue Native-PAGE (BN-PAGE) technology [118-120], there is a growing awareness that the individual components of the ETC may actually exist as large macromolecular assemblies, or so-called supercomplexes (Fig. 1.1B). Evidence accumulated from functional and structural studies now supports the existence of these supramolecular assemblies, which includes oxygen consumption characteristics [121], metabolic flux control analysis [122-127], and three-dimensional structures of the $I_1III_2IV_1$ supercomplex (an assembly comprised of a copy of complex I, dimeric

complex III and a copy of complex IV) from bovine heart mitochondria at relatively good resolution (~ 20 Å) [128, 129].

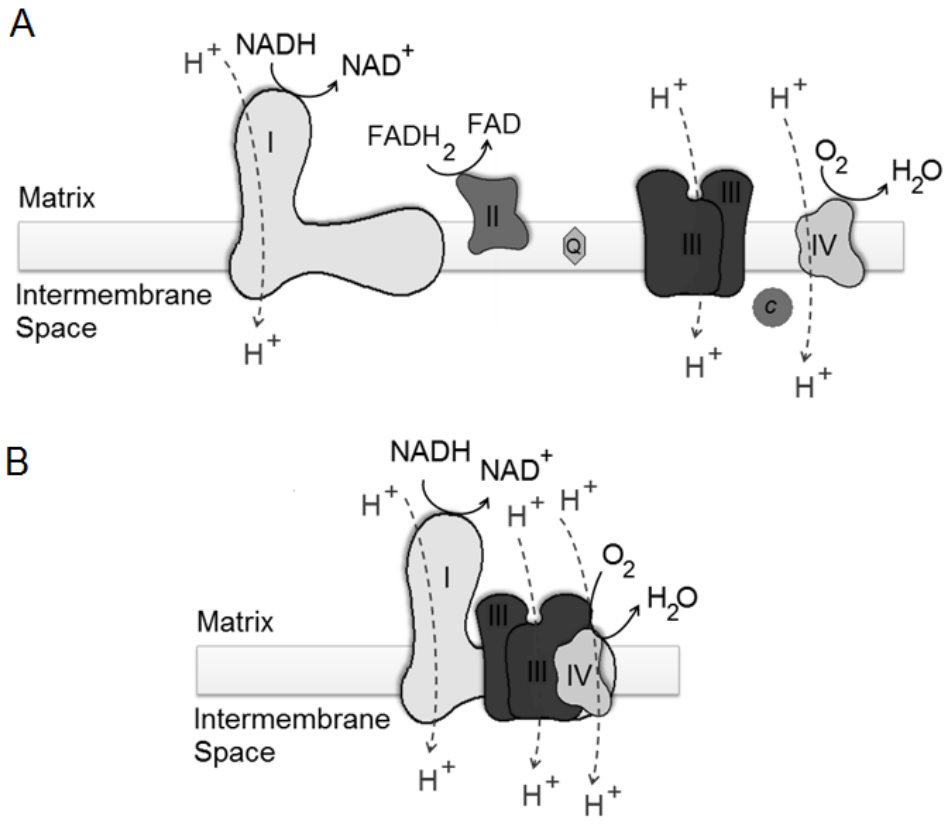

Figure 1.1 Different views of the organization of the mitochondrial ETC. (A) According to the random collision model, mobile electron carriers (e.g. cytochrome *c* and coenzyme Q) mediate electron transfer between ETC complexes I, II, III and IV. (B) Supramolecular assembly of complexes I, III and IV into a respiratory supercomplex of stoichiometry $I_1III_2IV_1$.

Although there was initial skepticism whether supercomplexes were artifacts from the use of a non-ionic detergent (e.g. digitonin) in their isolation, further extensive characterization of supercomplexes from mitochondria of different sources (Table 1.1) appears to have mitigated most concerns regarding their biological existence. This characterization includes identification of striking variations in stoichiometries of components comprising supercomplexes in tissues from the same species [130] or from cells obtained from different human tissues (Table 1.1). Moreover, metabolic flux control analysis measuring the kinetic behavior of the mitochondrial ETC and the ubiquinone pool further provide supporting evidence for supercomplex organization of the ETC [122-124, 126, 127]. Briefly, complexes I and III appear to be kinetically linked [122, 123, 127], while there is no association of complex II with any other complex. This latter result is in good agreement with BN-PAGE analysis using cardiac mitochondria [118, 130-132], including the characterization presented in Chapter 2, which indicates that supercomplexes are comprised of varying stoichiometries of complexes I, III, and IV. Finally, cryo-electron microscopic and tomographic studies independently show the existence of $I_1III_2IV_1$ supercomplexes [128, 129]. These studies show that the distance between binding sites of coenzyme Q at complexes I and III is only ~13 nm

[128, 129] and that the distance between binding sites of cytochrome *c* at complexes III and IV is ~10 nm [128, 129]. Such distances are much shorter than the minimum effective lengths determined for diffusion of coenzyme Q (37.9 nm) and cytochrome *c* (24.8 nm) during electron transfer by a random collision mechanism [115]. This suggests that supercomplexes offer a catalytic advantage of faster and more efficient electron transfer by limiting the overall distance between redox cofactors.

Finally, and in relation to the potential role that supercomplex destabilization plays in mitochondrial aging (see Chapter 3), supercomplexes may be necessary for general stability of the ETC. Moreno-Lastres et al. demonstrated that complexes III and IV are required for full assembly of complex I in mitochondria from human osteosarcoma cybrids [133]. Other studies show that removing complex III results in loss of the I_1III_2 supercomplex in human mitochondria from skeletal muscle [134], skin fibroblasts [135], and osteosarcoma cybrid cells [136]. Therefore, it is reasonable to theorize that not only does supercomplex organization mediate respiratory activity, but these macromolecular assemblies also regulate stability of individual components of the ETC.

Table 1.1 Major symmetric cardiolipin species and electron transport supercomplexes in mitochondria.

Source of mitochondria	Symmetric cardiolipin [a] (% of total)	Refs.	Supercomplexes [b]		Refs.
			I_1III_2	$I_1III_2IV_N$	
Heart					
Bovine	$(18:2)_4$ (70)	[137]	+	+	[118]
Rat	$(18:2)_4$ (77)	[112]	+	+	[143]
Dog	$(18:2)_4$ (77)	[138]	+	+	[144]
Liver					
Rat	$(18:2)_4$ (57)	[137]	+	—	[130]
Mouse	n.d.		+ **(V_1)** [c]	+ **(II_1)**	[121]
Skeletal muscle					
Rat	$(18:2)_4$ (73)	[112]	+	+	[130]
Human	$(18:2)_4$ (79)	[139]	+	+	[134]
Cell line					
C2C12	n.d.		+ **(V_1)**	+ **(II_1)**	[145]
HeLa	n.d.		+	+	[146]
HEK-293	n.d.		+	+	[147]
HL-60	n.d.		—	+	[148]
PBMC (human)	n.d.		—	+	[148]
Lymphoblasts (human)	$(18:1)_4$ (32)	[69, 140]	+	+	[149]
Skin fibroblasts					
Mouse	n.d.		+	+	[150]
Human	$(18:2)_4$ (30)	[141]	—	+	[151]
Lung fibroblasts (mouse)	$(18:1)_4$ (n.d.)	[142]	—	+	[142]
Osteosarcoma cybrids (human)	n.d.		—	+	[136]
TPC-1 (human)	n.d.		+	—	[123]

(Table continues on next page)

Table 1.1 *(continued)*

Source of mitochondria	Symmetric cardiolipin [a] (% of total)	Refs.	Supercomplexes [b]		Refs.
			I_1III_2	$I_1III_2IV_N$	
Other sources					
Brain cortex (rat)	n.d.		+	+	[154]
Kidney (rat)	$(18:2)_4$ (50)	[152]	+	+	[49]
Human placenta	$(18:2)_4$ (20)	[152]	+	+	[155]
S. cerevisiae	$(18:1)_4$ (31)	[69]	**III_2IV_1** [d]	**III_2IV_2**	[118]
N. crassa	$(18:2)_4$ (29)	[153]	+	+	[156]
C. elegans	n.d.		+	+	[157]
P. anserina	n.d.		+	+	[158]
P. denitrificans	n.d.		—	**$I_1III_4IV_4$**	[159]
Plants					
Potato	n.d.		+	+	[160]
Spinach green leaves	n.d.		+	+	[161]
A. maculatum	n.d.		+	+	[162]
Arabidopsis	n.d.		+	—	[163]
Bamboo	n.d.		+ **(V_1)**	—	[164]
Maize	n.d.		+	—	[165]

[a] Molecular species are presented as the acyl-chains (parenthesis) and their corresponding stoichiometries (subscript).

[b] I_1III_2 denotes a supercomplex comprising a single copy of complex I and dimeric complex III. $I_1III_2IV_N$ denotes supercomplexes comprising a single copy of complex I, dimeric complex III, and variable (N=1 - 4) copies of complex IV.

[c] Roman numeral in parenthesis denotes additional OXPHOS complexes also reported as part of supercomplexes.

[d] Alternative mitochondrial supercomplexes, different than I_1III_2 and/or $I_1III_2IV_N$-type assemblies.

n.d. = not determined.

In summary, there is growing evidence that the ETC is actually assembled as a solid-state macromolecular assembly where defective supercomplex organization results in pathologies as varied as heart failure [138, 144], Barth syndrome [149], and Leigh syndrome [134, 135].

Because these pathological conditions are reminiscent of aging, it is plausible to hypothesize that alterations in supercomplexes are partly responsible for the loss of ETC function characteristic of the aged heart. To investigate this hypothesis, this dissertation mainly focused on the study of supercomplex organization and stability in subsarcolemmal (SSM) and interfibrillary (IFM) mitochondria isolated from cardiac tissue from young (3-5 months) and old (24-28 months) male Fischer 344 rats. BN-PAGE analysis of digitonin-solubilized mitochondrial membranes coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to investigate supercomplex organization in cardiac mitochondria. In Chapters 2 and 4, we present a characterization of ETC supercomplexes in SSM and IFM. Our results show that both SSM and IFM display supercomplexes comprised of various stoichiometries of complexes I, III and IV (never complex II), which typically organize as high mass (1500 – 2300 kDa) assemblies containing up to four copies of complex IV (i.e. $I_1III_2IV_N$-type supercomplexes). In Chapters 2 and 4, we also show that

ETC supercomplexes are destabilized with age in rat heart mitochondria. Furthermore, our results indicate that IFM display more robust supercomplex deficits than SSM. In addition, a study of the mitochondrial factors that may be important for the age-associated deterioration of supercomplexes is presented in Chapters 3 and 4. Potential implications of these results are discussed in Chapter 3, based on both the theoretical and experimental evidence suggesting that supercomplex destabilization is part of mitochondrial aging.

Results presented in this dissertation constitute a characterization of age-associated alterations in the macromolecular organization of the IMM components that could partly explain the loss of mitochondrial oxidative capacity that affects the aging heart.

1.1.5 Fatty acid-driven bioenergetics in the aging heart

Age-associated changes in myocardial aerobic metabolism

Fatty acids constitute the primary fuel of the heart [18, 166]. However, the aging myocardium displays a decreased capacity to utilize fatty acids as the main energy substrate [44, 107, 167, 168]. In particular, cardiac fatty acid oxidation has been reported to decline with age in rodents

[107, 168, 169] and also in humans [44]. Whereas this decline in fatty acid-supported metabolism appears to be compensated by an increase in glucose utilization [44, 107], such a metabolic shift seems to cause abnormal lipid accumulation [170]. Because myocytes have limited means for exporting fatty acids in the form of triacylglyceride [171], lower fatty acid oxidation may shunt lipids into non-oxidizing metabolic pathways and/or lipid storage in the myocardium [170, 172, 173]. In its extreme, the age-associated decline in fatty acid-driven mitochondrial bioenergetics may thus initiate a form of myocardial lipotoxicity [174-176]. Therefore, it is important to understand the mechanism for lower fatty acid oxidation in the aging heart muscle.

While age-associated alterations in cardiac energy metabolism are undoubtedly multifactorial, several reports implicate carnitine palmitoyltransferase 1 (CPT1) as a key enzyme in the shift away from fatty acid oxidation [107, 177, 178]. CPT1, a mitochondrial protein and the rate-controlling enzyme for overall fatty acid β-oxidation, catalyzes the condensation of acyl-CoA with L-carnitine to form acyl-carnitine esters, which are subsequently transported into the mitochondrial matrix for further catabolism (Fig. 1.2) [179-181]. There is a consensus that CPT1 activity declines with age in heart and skeletal muscle [85, 107, 177, 182].

Moreover, down-regulation of CPT1 activity correlates with lipid accumulation and insulin resistance in rat skeletal muscle [182, 183]. Therefore, a plausible hypothesis is that lower CPT1activity is an underlying factor in the decline in fatty acid-supported myocardial bioenergetics. Nevertheless, the exact molecular mechanism involved in the age-related loss of fatty acid-driven bioenergetics is not fully understood.

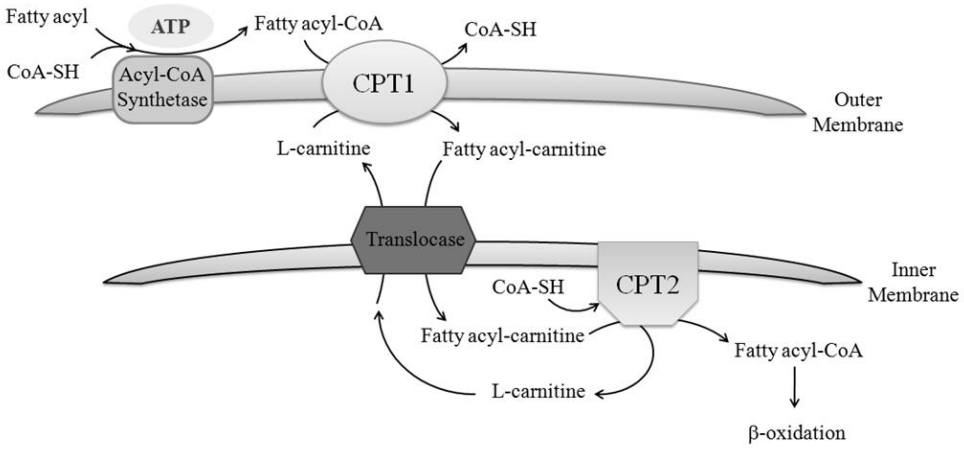

Figure 1.2 ATP-dependent activation of fatty acid molecules and their translocation into the mitochondrial matrix using the carnitine palmitoyltransferase (CPT) system. CoA-SH: coenzyme A.

As presented in Chapter 5, this dissertation also focused on characterizing alterations in CPT1 activity and fatty acid-dependent bioenergetics in two different populations of cardiac mitochondria, as important factors of mitochondrial decay in the aging rat heart. Results show that aging selectively decreases CPT1 activity in IFM by reducing enzyme catalytic efficiency for palmitoyl-CoA utilization without inducing significant alterations in kinetic parameters for L-carnitine.

L-carnitine metabolism and fatty acid-supported respiration in the aging heart

L-carnitine (Fig. 1.3) is a naturally occurring compound that, in concerted action with the enzymes of the carnitine palmitoyltransferase system (CPT1 and CTP 2) and the carnitine-acylcarnitine translocase of the IMM, facilitates the translocation of fatty acids into the mitochondrial matrix where they undergo degradation via β-oxidation (Fig. 1.2) [179-181]. While the liver and kidneys contribute to *de novo* synthesis of L-carnitine from 6-N-trimethyl-L-lysine, dietary uptake of L-carnitine plays a key role in maintaining its homeostasis in the body [184]. As fatty acids constitute the main energy substrate of the heart [18, 166], alterations in L-carnitine levels could severely impair mitochondrial fatty acid uptake and β-

oxidation in the myocardium. Specifically, age-related deficits of L-carnitine could be an important cause of impaired fatty acid utilization in the heart [44, 107, 168].

Because the content of L-carnitine has been reported to decline with age in humans [185, 186] and also in rats [187, 188], it is plausible to hypothesize that the aging lesion for fatty oxidation results from decrements in cardiac levels of L-carnitine. Furthermore, if carnitine levels indeed contribute to lower CPT1 activity, there is a rationale that general CPT1-mediated fatty acid oxidation can be remediated by increasing myocardial L-carnitine content.

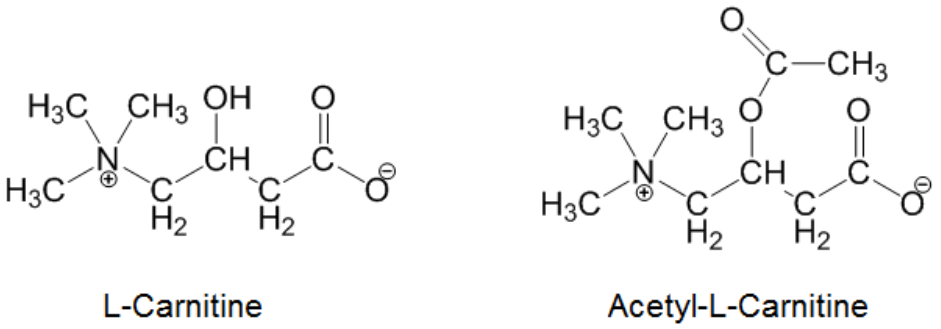

Figure 1.3 L-Carnitine and its acetylated form (ALCAR).

In Chapter 5, we present a characterization of age-related changes in the levels of both L-carnitine and acyl-L-carnitine, and the role they play in the loss of fatty acid-supported bioenergetics in the rat heart. Also, because cardiac mitochondrial bioenergetics in aged rats has been improved following dietary supplementation with the L-carnitine analogue, acetyl-L-carnitine (ALCAR) (Fig. 13) [86, 102, 103, 189], we investigated the potential mechanism(s) how ALCAR improves fatty acid-driven bioenergetics in the aging heart.

As presented in Chapter 5, ALCAR supplementation restored CPT1 activity in heart IFM, but not apparently through remediation of myocardial levels of L-carnitine. Rather, ALCAR influenced enzyme activity over time, potentially by modulating conditions in the aging heart that ultimately affect palmitoyl-CoA binding and CPT1 kinetics.

1.2 Specific Hypotheses and General Approach

The following are the two main hypotheses investigated in this dissertation:

First, it is hypothesized that **alterations of the macromolecular organization of the mitochondrial ETC as supercomplexes are partly responsible for the age-related loss of cardiac ETC function**. The rationale for this hypothesis is that recent structural and functional studies show that supercomplex deficits are a characteristic feature of certain pathologies (e.g. heart failure or Barth syndrome) reminiscent of aging. In order to test this hypothesis, the following aspects related to the molecular organization of the IMM were examined:

1. ETC supercomplexes were characterized in two different populations of rat heart mitochondria. To this end, BN-PAGE analysis of mitochondrial membranes coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to investigate supercomplex organization in mitochondria from male Fischer 344 rats.

2. Potential age-associated deterioration of supercomplexes was investigated in SSM and IFM isolated from cardiac tissue from young (3-5 months) and old (24-28 months) rats.
3. Age-related changes in IMM proteins and certain characteristic phospholipids (e.g. cardiolipin) were investigated as potential causes of supercomplex deterioration in cardiac mitochondria. Specifically, LC-MS/MS identification of proteins assembled into supercomplexes and HPLC analysis of cardiolipin were used to characterize supercomplex deterioration and its potential cause(s) in the Fischer 344 rat heart.

Second, it is hypothesized that **the age-related impairment of cardiac fatty acid catabolism occurs, at least partially, through diminished levels of L-carnitine, which would adversely affect CPT1.** In support of this hypothesis, several studies have shown that feeding ALCAR to old rats reverses the age-associated loss of physical activity, mitochondrial bioenergetics, and most importantly, fatty acid utilization. Thus, these results suggest that elevating myocardial carnitine levels could be key to remediating the decline in fatty acid oxidation. In order to test this hypothesis, the following aspects related to fatty acid-driven bioenergetics were examined:

1. Age-related differences in palmitoyl-CoA-supported respiration were investigated in cardiac mitochondria. Specifically, oxygen consumption characteristics were monitored in IFM and SSM in the Fischer 344 rat heart.
2. Age-associated alterations in CPT1 activity were determined in SSM and IFM. To this end, catalytic activity and parameters related to enzyme kinetics were determined in SSM and IFM.
3. Age-related alterations in myocardial levels of L-carnitine and their potential effect on global CPT1 activity were assessed in the Fischer 344 rat heart. Specifically, we investigated the potential mechanism(s) how ALCAR improves mitochondrial bioenergetics in aged rat hearts.

1.3 Dissertation Scope and Limitations

This dissertation constitutes a characterization of both dysfunction of the machinery involved in electron transfer and energy transduction, and impaired fatty acid-driven respiration as underlying factors of mitochondrial decay in two mitochondrial populations in the aging rat heart. Particularly, age-related alterations in the supercomplex organization of the ETC were

investigated as one of the factors that may limit bioenergetic reserve in the aged myocardium. In addition, potential age-related changes to certain IMM constituents that could cause supercomplex deficits were also investigated. While the results presented in this dissertation indicate that supercomplex deficits occur in cardiac aging, partially mediated by changes in the lipid organization of the IMM; further work is warranted to elucidate the complete characteristics associated to the age-associated deterioration of supercomplexes. Moreover, potential implication(s) and consequences of altered supercomplex organization were not part of this dissertation and require further investigation as they represent key aspects to understanding the true implications of mitochondrial decay in cardiac aging. Finally, our characterization of the age-associated loss of CPT1 activity in IFM provides new knowledge as to how aging impairs fatty acid utilization in the rat heart. However, specific protein modification(s) that lead to the aging lesion in IFM CPT1 catalysis were not studied in this dissertation and should be further investigated. Additionally, our findings that ALCAR improves IFM CPT1 catalysis are also important as they support a potential therapy for maintaining cardiac bioenergetics in the aging heart. Nevertheless, these observations warrant more studies to discern the mechanisms involved in ALCAR-dependent remediation of CPT1 activity.

Chapter 2

Supercomplexes of the Mitochondrial Electron Transport Chain Decline in the Aging Rat Heart

Luis A. Gómez, Jeffrey S. Monette, Juan D. Chavez, Claudia S. Maier,

Tory M. Hagen

Archives of Biochemistry and Biophysics
490 (2009) 30–35

Elsevier Inc. (Corporate Office)
360 Park Avenue South,
New York, NY 10010

2.1 Abstract

Accumulation of mitochondrial electron transport chain (ETC) defects is a recognized hallmark of the age-associated decline in cardiac bioenergetics; however, the molecular events involved are only poorly understood. In the present work, we hypothesized that age-related ETC deterioration stemmed partly from disassociation of large solid-state macromolecular assemblies termed "supercomplexes". Mitochondrial proteins from young and old rat hearts were separated by Blue Native-PAGE, protein bands analyzed by LC-MALDI-MS/MS, and protein levels quantified by densitometry. Results showed that supercomplexes comprised of various stoichiometries of complexes I, III and IV were observed, and declined significantly ($p < 0.05$, $n = 4$) with age. Supercomplexes displaying the highest molecular masses were the most severely affected. Considering that certain diseases (e.g. Barth syndrome) display similar supercomplex destabilization as our results for aging, the deterioration in ETC supercomplexes may be an important underlying factor for both impaired mitochondrial function and loss of cardiac bioenergetics with age.

Keywords: aging; rat heart mitochondria; electron transport supercomplexes; blue native-polyacrylamide gel electrophoresis.

2.2 Introduction

Mitochondrial decay has been implicated as one of the principal underlying factors of aging [23, 28, 48, 190-192]. Both significant and subtle alterations in mitochondrial ultrastructure have been observed which correlate with lower electron transport efficiency, increased superoxide appearance, and oxidative damage [193-195]. Though certainly multifactorial in nature, it is clear that electron transport chain defects are part of the age-associated loss of mitochondrial function [25, 77, 82, 105, 196]. Activities of complexes I, III and IV reportedly decline with age [23, 25, 26, 77, 105] and many acute mitochondrial-derived diseases show similar, if not more severe, electron transport chain deficits than seen in aging [197-202]. Thus a central research focus has been to understand the cellular and molecular processes that ultimately lead to electron transport chain defects in mitochondria from aging tissues.

Four large protein complexes comprise the electron transport chain (ETC), which along with the F_1F_O-ATP synthase (complex V), constitute the machinery for converting metabolic energy transiently stored as reduced coenzymes into ATP. Until recently, the prevailing view was that the components of the ETC were distinct entities where rapid, random

collisions allowed electron transfer between complexes [114]. However, with the advent of blue native-PAGE (BN-PAGE) technology, there is a growing awareness that the individual components of the ETC may actually exist as large macromolecular assemblies, or so-called supercomplexes [118-120]. Several biochemical and biophysical lines of evidence support the existence of these supramolecular assemblies [120, 122, 156, 203-205], including a proposed three-dimensional structure for a macromolecule composed of complexes I, III and IV with a stoichiometry of 1:2:1, respectively [206]. Although physical evidence for mitochondrial supercomplexes now exists by a variety of techniques outside of BN-PAGE, the ramifications of such large protein supercomplexes on ETC function are not completely understood. In this regard, there have been no studies critically examining age-associated changes to mitochondrial supercomplex levels or alterations to their structural composition. Moreover, there is a paucity of knowledge as to the role that supercomplexes play in the aging mitochondrial phenotype. Therefore, the goal of the present work was to characterize age-dependent changes in supercomplex levels and composition. The aging rat heart was chosen for this analysis as this organ exhibits a significant mitochondrial-driven impairment in bioenergetics

[207, 208], and supercomplexes are readily observed in mitochondria from this tissue [130, 131].

Using BN-PAGE separation of membrane proteins and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, this report shows that rat cardiac mitochondria display robust levels of ETC supercomplexes, but most of these assemblies, particularly those of the highest molecular weight, decline significantly with age. Thus, age-associated alterations in certain subtypes of supercomplexes must be considered as part of the etiology of mitochondrial alterations in the aging rat heart.

2.3 Materials and Methods

2.3.1 Materials

6-Aminohexanoic acid and digitonin were purchased from Acros-Organics (Morris Plains, NJ, USA), and n-dodecyl-β-D-maltoside from Calbiochem (San Diego, CA, USA). Sucrose, D-mannitol, MOPS, Tricine, Tris HCl, Bis-Tris, subtilisin A, acetonitrile, ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), KCl and other salts were from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were reagent

grade. Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). Coomassie G-250 and Bio-Safe Coomassie-G250 were from BioRad (Hercules, CA, USA). Mitochondrial complexes were detected using monoclonal antibodies against the following subunits: 39 kDa of complex I, 70 kDa of complex II, core 2 of complex III, subunit I of complex IV, and subunit α of complex V (MitoSciences, Eugene, OR, USA).

2.3.2 Ethical treatment of vertebrate animals

Young (5 months) and old (24 months) male Fischer 344 rats were obtained from the National Institute on Aging animal colonies. The animals were housed in approved facilities at the Linus Pauling Institute at Oregon State University and maintained by the Department of Laboratory Animal Resources. All procedures and animal handling were in keeping with approved institutional animal care and use guidelines.

2.3.3 Isolation of mitochondria

Heart interfibrillar mitochondria from young and old rats were isolated according to the protocol described by Palmer et al. [209]. One noted change from this method is the use of subtilisin A instead of nagarse

to release mitochondria from the myofibrils, as the latter compound is no longer commercially available. All steps of the isolation were performed on ice or at 4ºC. Protein content was determined by the Lowry method using bovine serum albumin as the standard (total protein kit from Sigma-Aldrich, St. Louis, MO, USA).

2.3.4 Separation of electron transport supercomplexes by BN-PAGE

Separation of electron transport supercomplexes was performed using BN-PAGE according to the protocol described by Wittig and Schägger [210, 211]. Mitochondrial membranes were solubilized with digitonin in a buffer composed of 750 mM 6-aminohexanoic acid, 50 mM Bis-Tris and 0.5 mM EDTA, pH 7.0 at 4ºC. A digitonin-to-protein ratio of 8:1 (w/w) was empirically determined to be optimal for solubilizing mitochondrial membranes but maintaining mitochondrial supercomplexes. Following solubilization, samples were centrifuged for 20 min at 12000 × g at 4ºC. Coomassie G-250 was added to the resulting supernatants using a detergent-to-dye ratio of 8:1 (w/w). Proteins (100 μg aliquots per lane) were separated on a NativePAGE Novex 3-12% Bis-Tris gradient gel (Invitrogen, Carsbad, CA, USA). In order to prevent an artifactual difference in the density of protein bands due to the position of lanes in the

gel, samples were loaded following an alternating sequence between mitochondria from young versus old animals. Following electrophoresis, protein bands were visualized using Bio-Safe Coomassie-G250. A gel imaging system (Alpha Innotech, San Leandro, CA, USA) was used to digitize gel images and the densitometric in-gel quantification of bands was performed using ImageJ (National Institutes of Health).

2.3.5 Separation of individual electron transport complexes by BN-PAGE

In order to control for differences in protein-loading as well as to establish a means to quantify the levels of individual ETC complexes, n-dodecyl-β-D-maltoside (DDM) was used to solubilize mitochondrial membranes. Solubilization with DDM allows for separation of individual complexes rather than retaining ETC supercomplexes [118, 210]. A DDM-to-protein ratio of 2:1 (w/w) was empirically determined to optimally separate ETC complexes in their individual forms. Mitochondrial membranes were solubilized in a buffer composed of 750 mM 6-aminohexanoic acid, 50 mM Bis-Tris and 0.5 mM EDTA, pH 7.0 at 4ºC. With the exception of DDM for membrane solubilization, all other conditions for BN-PAGE were the same as described for separation of electron transport supercomplexes (see above).

2.3.6 Identification of mitochondrial supercomplexes by nano-LC matrix assisted laser desorption ionization tandem mass spectrometry (MALDI-MS/MS)

Identification of proteins comprising individual bands on BN-PAGE gels were made using LC-MALDI-MS/MS analysis according to an adapted protocol from the method described by Fandiño et al. [212]. Prior to MALDI-MS/MS analysis, peptides were separated by nano-LC (column: PepMap 100, C-18, 3 μm, 100 Å, 75 μm i.d. x 15 cm, Dionex Corporation, Sunnyvale, CA, USA. Flow rate: 260 nl/min) and were spotted on 144-well MALDI target plates. Analysis of peptides was performed using a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Carlsbad, CA, USA). MASCOT (Matrix Science, Boston, MA, USA) was used to search the MS/MS data against the Swiss-Prot database (taxonomy rodentia) from which protein subunits of the oxidative phosphorylation system (OXPHOS) complexes were identified. Protein hits were considered significant with a significance threshold of $p < 0.05$ for peptide fingerprint search (i.e. a peptide match has less than 5% probability of being a random event), using an ion cut off score equal to 20. Searching

of a decoy database resulted in a false positive discovery rate of less than 5%.

2.3.7 Identification of mitochondrial complexes by Western blot

Following the electrophoretic separation of proteins (30 μg of total protein added per lane), gels were transferred onto PVDF membranes (Immobilon Millipore, Billerica, MA, USA). Immunodetection of each OXPHOS complex was independently performed. Mitochondrial complexes were detected using monoclonal antibodies against the following subunits: 39 kDa of complex I, 70 kDa of complex II, core 2 of complex III, subunit I of complex IV, and subunit α of complex V.

2.3.8 Statistical analysis

All results were analyzed using a two-sided two-sample *t*-test. Differences were considered statistically significant at $p < 0.05$. When performing the search of the MS/MS data using MASCOT, peptide matches were considered significant with a significance threshold of $p < 0.05$ for peptide fingerprint search (i.e. a peptide match has less than 5% probability of being a random event).

2.4 Results

2.4.1 Levels of individual ETC complexes do not change in the aging heart

Figure 2.1A shows the separation pattern of proteins by BN-PAGE of DDM-solubilized rat heart interfibrillar mitochondria. From the protein separation achieved, individual OXPHOS complexes were identified in mitochondria isolated from both young and old rat hearts. To investigate potential age-related differences in the abundance of electron transport complexes, protein content of the Coomassie-stained bands was analyzed using standard densitometric methods (see Materials and Methods). Because complex II is not associated with supercomplexes in cardiac mitochondria [118, 122, 131, 206], it was chosen as a means to normalize against differences in protein loading. As shown in Figure 2.1B, no significant differences ($p > 0.05$) in the levels of complexes I, III and IV were evident in mitochondria isolated from old rats relative to young controls. Because of the potential for contaminating proteins to comigrate with ETC complexes, Western blot analysis was also used as to confirm the densitometric results (Fig. 2.1C). Using antibodies specific for each complex, no significant differences ($p > 0.05$) in the levels of any complex

were evident with age (Fig. 2.1D). Thus, aging does not result in changes to levels of any individual electron transport complex.

2.4.2 Separation of rat cardiac mitochondrial supercomplexes by BN-PAGE and LC-MALDI-MS/MS analysis

Solubilization of mitochondrial membranes with digitonin versus DDM resulted in a more complex separation profile of proteins by BN-PAGE. Protein bands of very high mass were observed (S_1 to S_4, Fig. 2.2), which suggested the presence of supramolecular assemblies. Careful removal of these protein bands followed by LC-MALDI-MS/MS analysis showed that complexes I, III and IV were constituents of these high-mass protein bands (Fig. 2.2). Thus, electron transport supercomplexes were identified in heart interfibrillar mitochondria. As multiple high molecular weight protein bands were observed using BN-PAGE, our data further suggest the presence of supercomplexes composed of complexes I, III and IV with differing stoichiometries. Another protein assembly comprising complexes I and III was also observed (designated "S_C" in Fig. 2.2). There is a potential for complex V to also be associated with this particular supercomplex, but because of poor band separation by BN-PAGE, it is not currently possible to determine whether complex V is an integral part of the S_C assembly or merely comigrates with it on the gel. Regardless, the S_C

protein complex displayed the lowest molecular mass with respect to the other identified supercomplexes. As noted previously, no association of complex II with other complexes was observed, indicating that it is not part of any respiratory supercomplex in cardiac mitochondria [118, 122, 131, 206] (Fig. 2.2).

In addition to supercomplex identification, LC-MALDI-MS/MS analysis also denoted protein bands consisting solely of complexes I, II, III or IV (Fig. 2.2). Separation of complex V as a single unit could not be optimally achieved using digitonin at the concentrations used. The identity of all proteins separated by BN-PAGE of digitonin-solubilized rat heart interfibrillar mitochondria was also confirmed using Western blot analysis of individual OXPHOS complexes (data not shown).

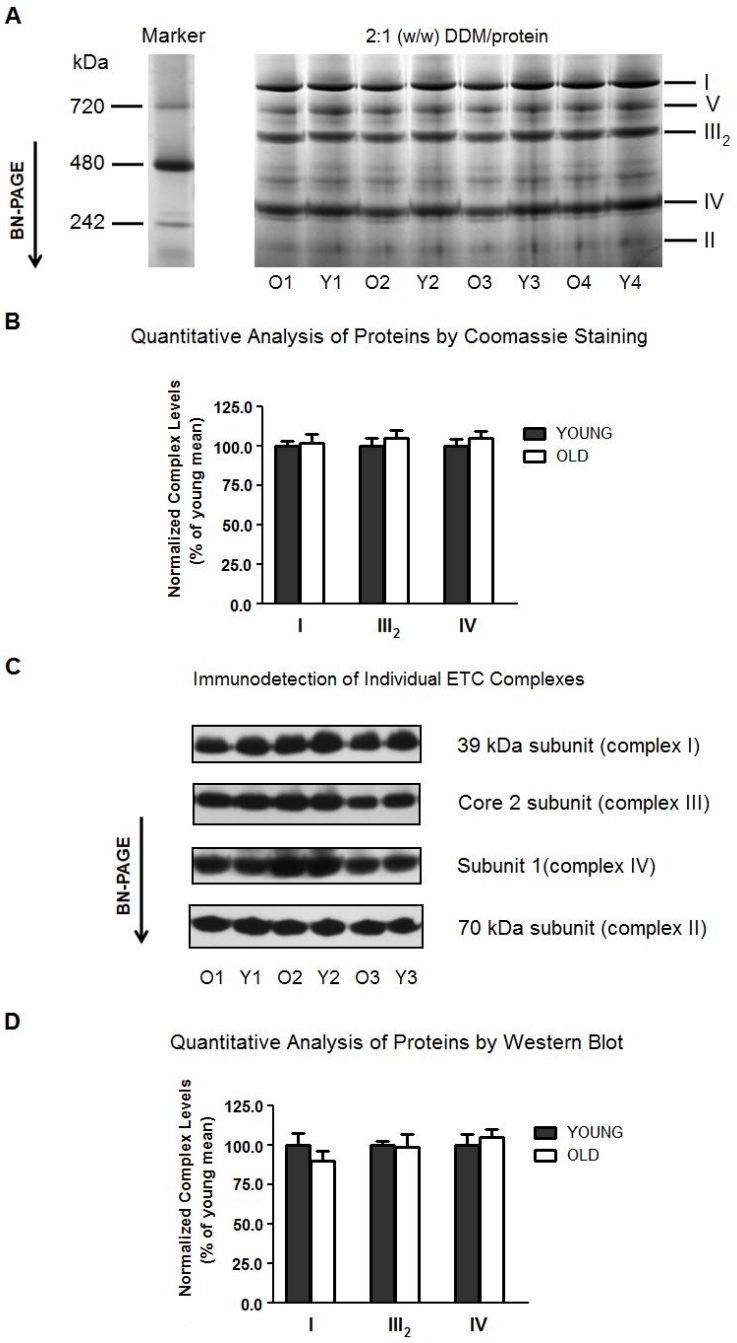

Figure 2.1 The levels of individual electron transport complexes of rat heart interfibrillary mitochondria do not change with age *(see legend on next page)*.

Figure 2.1 *(see previous page)* (A) Heart interfibrillar mitochondria were isolated from young (Y: 5 months, n = 4) and old (O: 24 months, n = 4) Fischer 344 rats, and membranes were solubilized with a DDM-to-protein ratio of 2:1 (w/w). NativeMark (Invitrogen, Carsbad, CA, USA) was used as a molecular weight standard for proteins separated by BN-PAGE. (B) Levels of complexes I, III and IV were calculated using the density of Coomassie-stained proteins. In order to control for differences in protein-loading, the density of each complex was normalized to the density of complex II from the corresponding lane. (C) In separate experiments, mitochondrial proteins were separated by BN-PAGE as indicated in (A), and Western blot analysis was used for identification of individual electron transport complexes. Mitochondrial complexes were detected using monoclonal antibodies against the following subunits: 39 kDa of complex I, 70 kDa of complex II, core 2 of complex III and subunit I of complex IV, as described in Materials and Methods (Section 2.3.7). (D) Levels of complexes I, III and IV were calculated using densitometric analysis after identification of proteins by Western blot. In order to control for differences in protein-loading, the density of each complex was normalized to the density of complex II from the corresponding lane. I, II, III_2, IV and V denote individual OXPHOS complexes. All results are presented as the mean ± SEM, and plotted as a percentage of the mean from young controls.

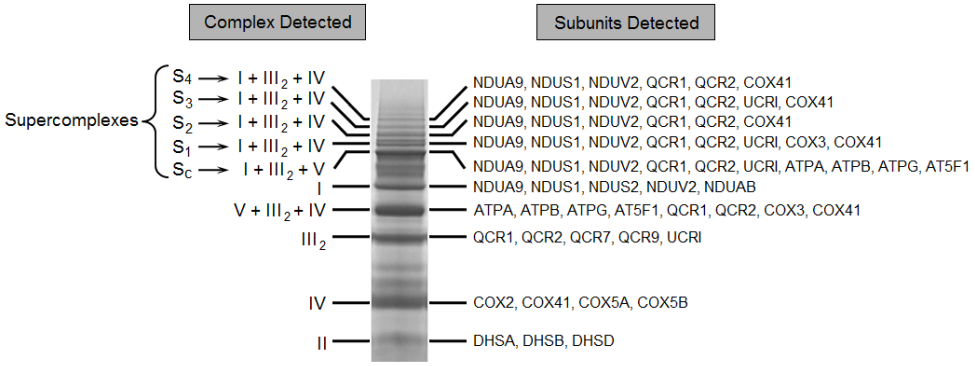

Figure 2.2 Identification of electron transport supercomplexes in rat heart interfibrillar mitochondria by LC-MALDI-MS/MS analysis. Mitochondrial proteins were separated on a 3-12% Bis-Tris gradient polyacrylamide gel by one-dimensional BN-PAGE. Individual mitochondrial OXPHOS complexes were detected using LC-MALDI-MS/MS analysis. The most representative protein subunits detected for each OXPHOS complex are displayed. I, II, III_2, IV and V denote individual OXPHOS complexes. S_C to S_4 indicate different mitochondrial supercomplexes. All proteins were identified by MASCOT (Matrix Science, Boston, MA, USA) using the parameters described in Materials and Methods (Section 2.3.6). Subunits are named according to the designation approved by the Protein Knowledgebase (UniProtKB) (http://www.uniprot.org/). Results are representative of three mitochondrial preparations.

2.4.3 Mitochondrial supercomplexes are diminished in the aging heart

The same overall separation pattern of proteins was observed by BN-PAGE of digitonin-solubilized cardiac mitochondria isolated from both young and old rats (Fig. 2.3A). Nevertheless, with age, most of the electron transport supercomplexes declined in heart interfibrillar mitochondria (S_1 to S_4, Fig. 2.3A). In order to quantify the extent of such an age-related loss,

supercomplexes were collectively examined. Summing the normalized densities of protein bands corresponding to S_1, S_2, S_3 and S_4 (Fig. 2.3A) revealed that interfibrillar mitochondrial supercomplexes collectively diminished by 15% relative to young controls ($p < 0.05$, $n = 4$) (Fig. 2.3B).

Although practically all the identified high molecular weight assemblies were comprised of complexes I, III and IV (Fig. 2.2), not all these supercomplexes deteriorated to the same degree as a function of age. As shown in Figure 2.3C, the assemblies displaying the highest molecular masses underwent the largest age-related declines. Relative to young controls, supercomplexes S_4, S_3 and S_2 declined by 21%, 25%, and 13%, respectively ($p < 0.05$, $n = 4$) (Fig. 2.3, A and D). There was a trend ($p < 0.07$, $n = 4$) for the protein band designated S_1 to also decline with age, but no significant difference ($p > 0.05$) in the multicomplex assembly named S_C was observed. On the other hand, no significant differences ($p > 0.05$) in the free forms of complexes I and IV were detected; however, complex III was diminished by ~12% ($p < 0.05$, $n = 4$), relative to young controls (Fig. 2.3D). Thus, aging results in lower levels of mitochondrial supercomplexes without a concomitant loss in individual components of the ETC.

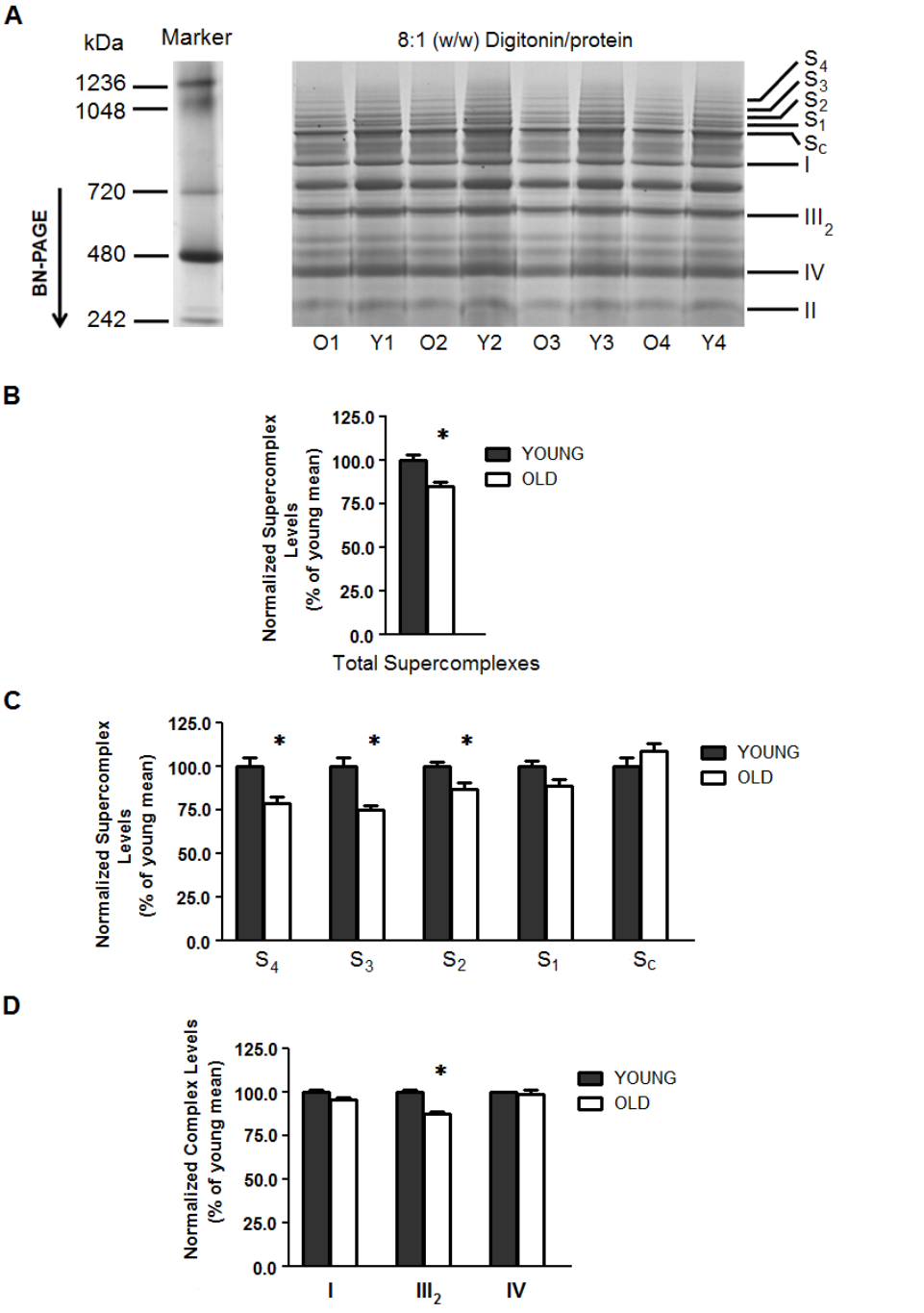

Figure 2.3 Mitochondrial electron transport supercomplexes are diminished in the aging rat heart *(see legend on next page)*.

Figure 2.3 *(see previous page)* (A) Heart interfibrillar mitochondria were isolated from young (Y: 5 months, n = 4) and old (O: 24 months, n = 4) Fischer 344 rats, and solubilized with a digitonin-to-protein ratio of 8:1 (w/w). NativeMark (Invitrogen, Carsbad, CA, USA) was used as a molecular weight standard for proteins separated by BN-PAGE. (B) From each lane, the total levels of supercomplexes were estimated by summing the densities of the four protein bands corresponding to supercomplexes (i.e. S_1-S_4). In order to control for differences in protein-loading, the density of each supercomplex was normalized to the density of complex II from the corresponding lane. (C) Levels of mitochondrial electron transport supercomplexes. (D) Levels of electron transport complexes in a free form. S_1-S_4 indicate different mitochondrial electron transport supercomplexes. I, II, III_2, IV and V denote individual OXPHOS complexes. All results are presented as the mean ± SEM, and plotted as a percentage of the mean from young controls. n = 4; *$p < 0.05$ compared to young controls.

2.5 Discussion

BN-PAGE was used to separate but maintain mitochondrial proteins in a native architecture [118, 131]. Using this technique, we observed both individual electron transport complexes as well as high molecular weight protein bands previously identified as supercomplexes [118, 131]. Additionally, both LC-MALDI-MS/MS analysis and immunodetection were employed to confirm that these high molecular weight protein bands were indeed macromolecular assemblies of individual ETC components. Thus, analytical techniques of sufficient resolving power were used to investigate age-associated changes to mitochondrial supercomplexes. Nevertheless, we

recognize that even though BN-PAGE has been instrumental in showing that individual ETC complexes assemble into supercomplexes, the necessity of membrane solubilization with specific detergents may cause artifactual protein aggregates which in turn affect interpretation of data.

It is clear from our data that significant age-related losses in most identified supercomplexes occur, with those of the highest molecular masses the most severely diminished. As the profile of mitochondrial proteins separated by BN-PAGE was not affected with age (Fig. 2.3A), it is reasonable to assume that the loss of supercomplexes is not caused by any age-related deficit of constituent subunits of ETC complexes. However, because the present work constitutes the first characterization of age-dependent changes in supercomplexes, we are aware that BN-PAGE as a technique might produce variations in results when using mitochondrial preparations from young versus old rats. Therefore, the potential contribution of other factors such as a difference in the protein yield between heart mitochondria from young and old animals during solubilization of membranes for BN-PAGE analysis cannot be excluded. The mechanism(s) for the general deterioration in supercomplexes are currently unknown and were not explored in the present work. However, as complex IV is a constituent of all supercomplexes in rat heart mitochondria

and also the last component to be incorporated into them [121, 134], it is enticing to speculate that age-related supercomplex decay is connected with alterations in complex IV. In this regard, Oswald et al. established an association with complex IV and destabilization of supercomplexes when they showed that knockdown of *COX17* in HeLa cells resulted in supercomplex loss [146]. Moreover, D'Aurelio and colleagues also observed supercomplex destabilization in human mtDNA cybrids containing a mutation in the cytochrome *c* oxidase subunit 1 gene (*MT-COX1*) [136]. Finally, we previously showed that complex IV activity declined with age, which correlated with extensive oxidative modification of this complex [26]. These latter results, together with our present data, may therefore establish a rationale for oxidative protein modification of complex IV and decline in supercomplex assembly. We are presently exploring the connection between alterations in complex IV components and the age-related loss of supercomplexes.

While the age-associated loss of interfibrillary mitochondrial supercomplexes was significant, the degree of diminishment was relatively modest. Thus it will be important to ascertain how these changes are part of the aging mitochondrial phenotype. For perspective, supercomplex deterioration has been observed for both Barth syndrome [149] and in a

patient with classic Leigh syndrome [134]. Despite severe mitochondrial ultrastructural and respiratory chain defects evident in both cases, only relatively subtle declines in supercomplex levels were noted (~20% for Barth syndrome, [149]). Additionally, in an acute heart failure model in dogs, disassembly of mitochondrial supercomplexes only reached 50% relative to control animals [144]. Thus, the apparent subtle loss of supercomplex levels observed in our present study (i.e. ~15%) is not far below that seen in other pathologies connected to mitochondrial dysfunction. Therefore, even seemingly small deficits in mitochondrial supercomplex levels may significantly impact ETC function. In this regard, our results may at least partly account for the decline in oxidative capacity evident in the aging rat heart [23, 47, 105, 207, 208].

The data presented herein are also important for the so-called "respiratory string model" proposed by Schägger and colleagues [118-120]. This model postulates that the ETC exists as long super-assemblies that wrap the cristae and efficiently transfer electrons to O_2. If this model is correct, then the supercomplexes noted by BN-PAGE are themselves fragments of even higher ordered ETC strings [120, 213]. Our results interpreted in relation to this model indicate that aging leads to respiratory string destabilization in rat heart mitochondria. A schematic representation

of this scenario is depicted in Figure 2.4. An extension of this concept further suggests that if electron transport supercomplexes limit formation of reactive oxygen intermediates as previously suggested [118], age-associated destabilization of respiratory strings would promote production of oxidants and oxidative damage, which have been observed in isolated rat cardiac myocytes in general [207], and interfibrillar mitochondria in particular [26, 208]. Nevertheless, this interpretation requires further investigation as the current evidence in support of supercomplexes has not been sufficient to mitigate all doubts about the physiological existence of such a supramolecular organization of the ETC.

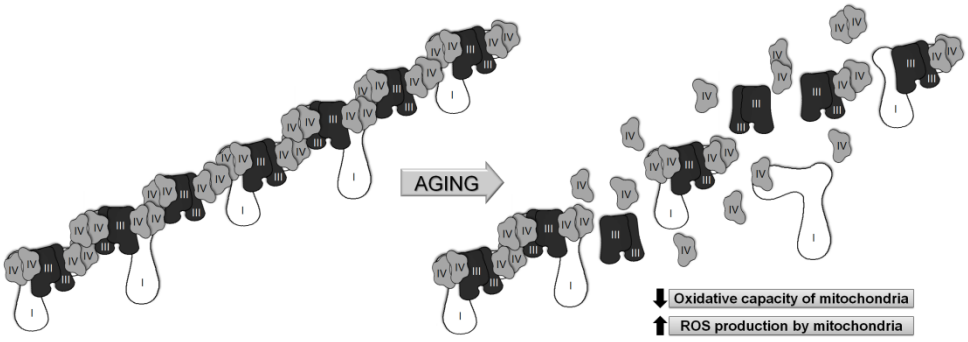

Figure 2.4 Schematic representation of the age-related destabilization of the mitochondrial ETC "respiratory string". "ROS" denotes reactive oxygen species.

Despite the implications for deficits to ETC function, the precise mechanism(s) involved in the age-associated loss of rat heart mitochondrial supercomplexes have yet to be elucidated. As individual ETC components do not decline with age, our data suggest that either supercomplex formation is impaired or the rate of decomposition is accelerated. Aside from changes to complex IV, evidence has accumulated suggesting that cardiolipin promotes supercomplex assembly [149, 214, 215]. Because cardiolipin has been observed to decline and/or its acyl side-chain composition changes with age [105, 113], it is tempting to think that age-related deficits in cardiolipin content may be responsible for a destabilization of supercomplexes. However, Hoppel and colleagues provided evidence that interfibrillary mitochondria of the aging rat heart have no age-associated decrements in cardiolipin, belying previous reports to the contrary [109]. Thus, additional work is warranted in order to define the role that cardiolipin plays in age-associated supercomplex deficits.

Another possibility contributing to loss of supercomplexes may result from an age-related alteration of the mitochondrial proteome. In this regard, prohibitins have become very interesting targets since such proteins have been observed to regulate the assembly of the ETC [216], and appear to play an important role during aging [217-219]. We are currently

examining the potential for both cardiolipin and/or age-associated alterations to ETC assembly to constitute the mechanism(s) associated with supercomplex degradation with age.

Chapter 3

Destabilization of Supercomplexes in Aging: Mechanism(s) and Potential Implications for Mitochondrial Function

Some sections of this chapter are excerpts from the following review article:

Age-related decline in mitochondrial bioenergetics: Does supercomplex destabilization determine lower oxidative capacity and higher superoxide production?

Luis A. Gómez and Tory M. Hagen

Seminars in Cell and Developmental Biology
23 (2012) 758– 767

Elsevier Inc. (Corporate Office)
360 Park Avenue South,
New York, NY 10010

3.1 Abstract

Mitochondrial decay plays a central role in the aging process. Although certainly multifactorial in nature, defective operation of the electron transport chain (ETC) constitutes a key mechanism involved in the age-associated loss of mitochondrial energy metabolism. Primarily, mitochondrial dysfunction affects the aging animal by limiting bioenergetic reserve capacity and/or increasing oxidative stress via enhanced electron leakage from the ETC. Even though the important aging characteristics of mitochondrial decay are known, the molecular events underlying inefficient electron flux that ultimately leads to higher superoxide appearance and impaired respiration are not completely understood. In this respect, we reported in Chapter 2 that supercomplexes deteriorate in aging rat heart mitochondria, which may be partly responsible for the age-related loss of cardiac ETC function. Because the underlying factor(s) of supercomplex deterioration in aged tissues are not fully known, this chapter focuses on how age-related alterations of the IMM constituents (e.g. ETC proteins and/or lipids) may be important factors of supercomplex destabilization in post-mitotic tissues in general and in cardiac mitochondria in particular. Additionally, this chapter focuses on the potential role(s) that age-

associated destabilization of supercomplex organization of the ETC (i.e. supercomplexes) may be important for development of the mitochondrial aging phenotype, particularly in post-mitotic tissues.

Keywords: aging; mitochondrial dysfunction; electron transport supercomplexes.

3.2 Introduction

Alterations of the components of the mitochondrial energy transduction system represent an important part of the etiology of mitochondrial decay and cellular dysfunction in aging tissues [27]. In particular, accumulation of age-related changes to both the structure and function of the electron transport chain (ETC) [23-25, 75] and alterations of the lipid milieu of the inner mitochondrial membrane (IMM) have been noted [28, 100, 106, 107]. Moreover, these changes to the IMM may contribute to decreased aerobic metabolism, increased generation of reactive oxygen species (ROS) and oxidative damage (for reviews see [39, 40, 56, 57]), and higher appearance of proapoptotic factors in the cytosol [50, 53, 54]. Nevertheless, the relationship between IMM structural alterations with age and their precise consequence to mitochondrial decay is only poorly understood. This is particularly true for the protein complexes of the ETC, whose overall structural organization is currently being revised from that of individual electron transport complexes in the IMM to large "supercomplexes" composed of differing stoichiometries [118-120]. Assembly and stability of supercomplexes appears to be highly important for regulation of mitochondrial bioenergetics as even slight losses in their

formation, as shown in Barth syndrome, correlate with severe cellular dysfunction [149]. Furthermore, supercomplex destabilization is the main underlying factor involved in the loss of mitochondrial bioenergetics in a canine model of acute heart failure [138, 144]. Thus, there is growing evidence that supercomplex destabilization plays a critical role in the progression of pathophysiologies where mitochondrial dysfunction has been detected. By analogy, a rationale exists that disintegration of supercomplexes is one of the characteristic features of age-related mitochondrial decay in post-mitotic tissues [132, 154, 220].

Particularly, results presented in Chapters 2 and 4 indicate that supercomplex levels decline with age in rat heart mitochondria. Moreover, our data show that mitochondria from different subcellular locations in the myocyte display different degrees of supercomplex destabilization in the aging heart. Nevertheless, it is not clear whether age-related alterations of the IMM components are an important cause of deterioration of mitochondrial supercomplexes. Thus, this chapter reviews potential factors that may regulate supercomplex organization and stability, with a particular focus on mitochondria from cardiac tissue. In an extension of such an analysis, this chapter also discusses how alterations to supercomplex

ultrastructure may be an important underlying facet leading to the mitochondrial aging phenotype.

3.3 Evidence of Supercomplex Destabilization in Aged Tissues

While only a limited number of studies have examined how aging affects supercomplexes, the information gleaned so far strongly suggests that the size and complexity of these assemblies diminish on an age basis. To our knowledge, we were the first to show supercomplex deterioration in cardiac mitochondria from old rats (Chapters 2 and 4, and [132]). Primarily, supercomplexes comprising the highest molecular weight (i.e. 1900-2300 kDa) assemblies declined to the greatest extent with age, although most of the electron transport supercomplexes showed some degree of loss (Chapters 2 and 4, and [132]). However, it is notable that supercomplex disintegration was not from age-associated decrements in a particular ETC component (Chapter 2 and [132]). In support of our work in the heart, Frenzel et al. also reported supercomplex destabilization in cortical tissue of aging rat brains [154]. This study also noted that $I_1III_2IV_N$ supercomplexes are particularly adversely affected [154]. However in contrast to these studies, Lombardi et al. noted an age-associated increase in $I_1III_2IV_N$

supercomplexes in rat skeletal muscle [220], which was hypothesized to be a compensation for the significant loss of the smaller I_1III_2 supercomplex. Finally, a recent report showed no age-associated alterations in supercomplex organization at all in rat kidney mitochondria [49], even though loss of State 3 respiration was detected [49]. Taken together, there appears to be varying degrees of age-dependent supercomplex disorganization where brain and heart mitochondria are the most adversely affected, and other tissues (e.g. skeletal muscle and kidney) have lesser or no supercomplex decrements. The reason(s) for this variability are not presently clear but could stem from tissue-specific factors related to supercomplex stabilization and/or levels of individual ETC components available for supercomplex formation. However, it is quite conceivable that part of the seeming variability for supercomplex destabilization may stem from the small number of studies performed to date and differences in analytical protocols and quantitative analysis used. Nevertheless, the identification of severe age-associated alterations in supercomplex levels, especially in post-mitotic tissue, warrants further analysis of both the causes and the consequences to mitochondrial function.

3.4 Major Factors that Alter Supercomplex Stability in Aging

The precise mechanism(s) involved in supercomplex destabilization have yet to be elucidated. However, genetic studies using yeast [214, 215, 221] as well as cryo-electron microscopy [128] are beginning to elucidate a role for lipid-protein interactions as a partial mechanism for this deterioration. For example, experiments on yeast mitochondria indicate that cardiolipin lowers electrostatic repulsion at the interface between complexes III and IV [221]. Moreover, the ETC complexes assembled as the $I_1III_2IV_1$ supercomplex are not in close contact with each other (2-5 nm apart) within the IMM [128], which indicates that lipid-protein interactions are key to maintaining supercomplex stability. In addition, there is a strong correlation between the levels of $I_1III_2IV_N$ supercomplexes and mitochondria from tissues displaying relatively high content (~70-80%) of cardiolipins with $(18:2)_4$-acyl side-chains (Figs. 3.1 and 3.2) (Table 1.1).

This association fits with observations that "symmetrical" cardiolipins containing similar acyl chains promote protein-protein interactions and ETC function [69, 222, 223]. Indeed, the aforementioned study by Frenzel et al. suggested that the age-related destabilization of brain cortical supercomplexes was mediated by altered cardiolipin content [154].

Thus, it is tempting to hypothesize that the oxidative damage of unsaturated cardiolipin side-chains that has been observed in the heart and brain from aged animals [77, 79] leads to supercomplex deterioration by altering binding of cardiolipin to ETC proteins. In support of this hypothesis, Diaz et al. recently reported that increased ROS generation destabilizes supercomplexes in mouse lung fibroblasts [224]. In this study, lower supercomplex levels correlated with decreased stability of complex I in an antimycin A-supported model of ROS generation. Nevertheless, it is not clear whether oxidatively damaged membrane lipids also contributed to supercomplex destabilization under the same conditions [224]. These studies provide a framework for investigating the role that IMM lipid composition plays in both supercomplex formation and destabilization with age.

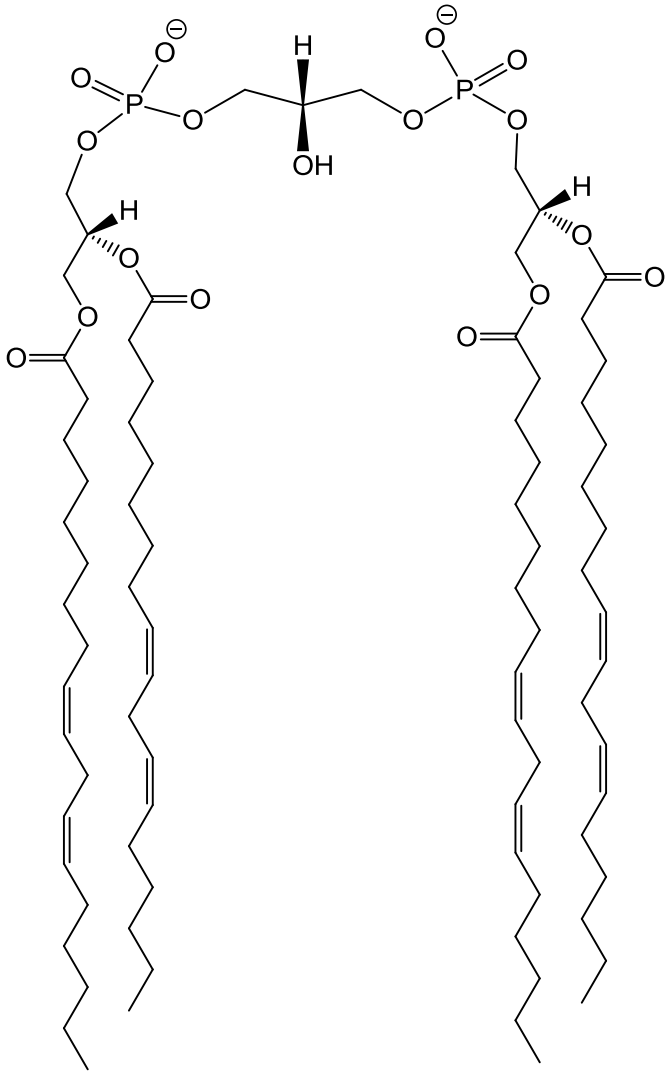

Figure 3.1 Tetralinoleoyl cardiolipin [$(18:2)_4$-cardiolipin], the predominant cardiolipin molecular species in cardiac mitochondria. Cardiolipin symmetry is due to the presence of linoleic acid (18:2n-6) in both positions of each diacylglycerol motif.

As shown in Figure 3.2, protein bands previously characterized as mitochondrial supercomplexes in the rat heart (see Chapter 2) were also resolved by BN-PAGE analysis of monkey heart mitochondria. It is noticeable; however, that while some fraction of complex I is found in the free form in the rat heart, all complex I was found as part of supercomplexes in monkey mitochondria (Fig. 3.2). Contrary to the results for myocardial tissue, high mass (1700-2300 kDa) assemblies containing up to four copies of complex IV (i.e. $I_1III_2IV_N$-type supercomplexes) were not detected by BN-PAGE of rat cardiac myoblasts (H9c2 cells), which only displayed a smaller supercomplex (S_C, 1500 kDa) comprised of complex I and dimeric complex III (i.e. I_1III_2 supercomplex). Thus, differences in ETC supercomplexes are evident even among mitochondria from highly homologous sources.

It is also interesting that these apparent differences in supercomplex organization were found to parallel, at least in part, differences in the content of $(18:2)_4$-cardiolipin. Particularly, changes in the proportion of $(18:2)_4$-cardiolipin relative to $(18:2)_3(18:1)_1$-cardiolipin, an intrinsic indicator of the content of symmetrical cardiolipin in cardiac mitochondria [112], were observed among mitochondria from different sources. Results showed that while this ratio between cardiolipins is quite similar in the

Rhesus monkey (5.48 ± 0.33, n = 24; unpublished results) and the Fischer 344 rat (4.78 ± 0.41, n = 4, see Chapter 4), it is much higher than the corresponding value observed by Schlame et al. in H9c2 cells (~0.5) [112]. Thus, it is enticing to hypothesize that $(18:2)_4$-cardiolipin, rather than global levels of cardiolipin, may be an important factor for stabilization of ETC supercomplexes, at least in cardiac mitochondria. However, these results also indicate that organization and stability of supercomplexes is a concerted mechanism that involves different components of the IMM (e.g. ETC proteins) and is not limited to the effect of cardiolipin.

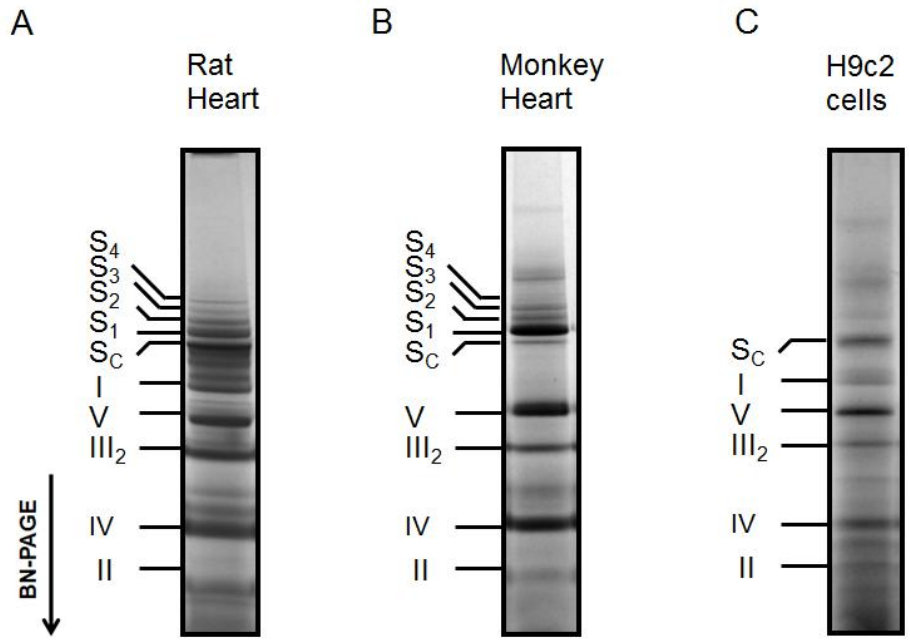

Figure 3.2 Mitochondrial supercomplexes in the mammalian heart and cardiac myoblasts (H9c2 cells). Mitochondria were isolated from rat (A) and monkey (B) hearts, and H9c2 cells (C). Supercomplexes were separated by BN-PAGE upon solubilization of isolated mitochondria with digitonin (8 g detergent/g protein). I, II, III_2, and IV denote individual forms of complexes of the electron transport chain, and V indicates to the F_1F_O-ATPase complex. S_C and S_1-S_4 denote I_1III_2 and $I_1III_2IV_N$ (N = 1-4) supercomplexes. Results are representative of at least three independent experiments. Monkey left ventricles were obtained from Dr. Rafael de Cabo (National Institue on Aging).

In addition to lipid involvement, disintegration of supercomplexes may also stem from alterations to proteins of the IMM. In particular, complex IV is a key player in supercomplex formation or, alternatively, supercomplex destabilization with age. In fact, it has been reported that complex IV activity declines in aged rat heart interfibrillar mitochondria [23, 26, 87], which correlates with extensive oxidative modification of this complex [26]. Moreover, alterations of several complex IV subunits are associated with defective assembly of $I_1III_2IV_N$ supercomplexes in mitochondria from human cells [134, 136, 146, 147], rodents [121, 150], and *C. elegans* [157]. For example, phosphorylation of complex IV results in the loss of respirasomes in a canine model of acute heart failure [138]. Therefore, oxidative or posttranslational modifications of complex IV may regulate supercomplex stability in cardiac mitochondria [138]. Finally, studies from multiple laboratories have supplied exciting new information showing that specific protein factors are involved in supercomplex assembly [145, 225, 226]. Rcf1 (Respiratory Supercomplex Factor 1) associates with complex IV and is required for supercomplex stabilization in yeast mitochondria. Moreover, Chen et al. showed that defective organization of $I_1III_2IV_N$ supercomplexes results from siRNA-mediated knock down of the rcf1 mammalian homologue, *HIG2A* (hypoxia inducible

gene 1, family member 2A), in C2C12 myoblasts [145]. Thus, HIG2A or other as yet unidentified proteins may also regulate supercomplex stability, particularly in post-mitotic tissues (e.g. the heart and brain) that show the most severe supercomplex disintegration with age.

In summary, both lipid factor(s) and proteins associated with the IMM may be involved in the assembly and maintenance of mitochondrial supercomplexes (Fig. 3.3). While little evidence currently exists for a precise role of any one factor in the loss of supercomplexes with age, nevertheless, it is noteworthy that both cardiolipin structural alterations and protein oxidation markedly increases on an age basis, particularly in mitochondria where supercomplexes deteriorate most appreciably. Chapter 4 focuses on identifying specific biological factors that may play an important role in supercomplex destabilization in the aging rat heart.

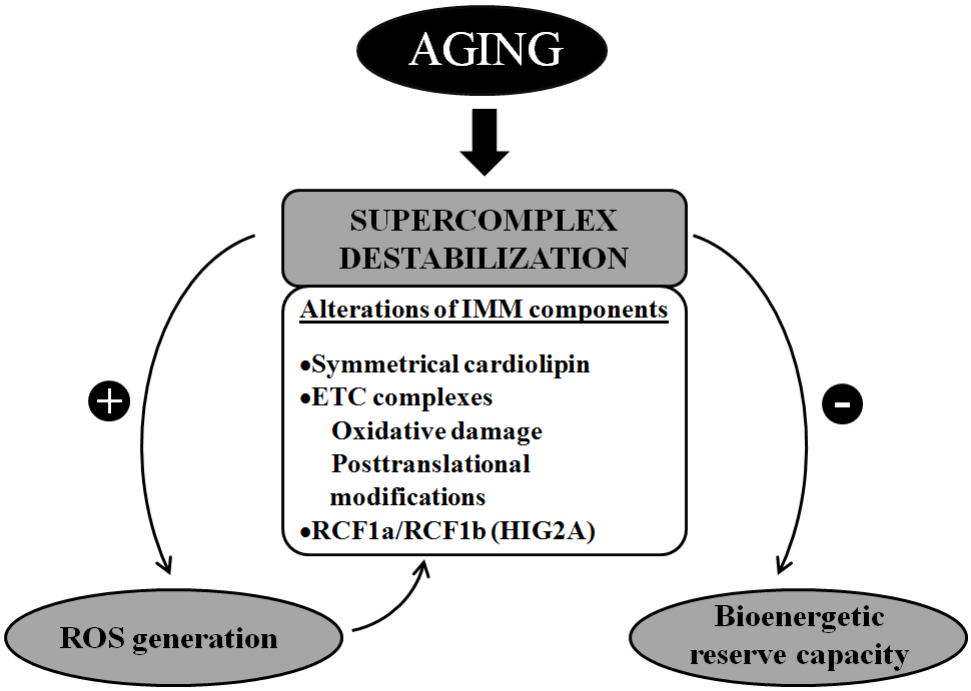

Figure 3.3 Supercomplex destabilization and mitochondrial decay in aging. Age-related alterations of the IMM components are presented as important factors affecting stability of supercomplex organization. Potential consequences of supercomplex destabilization on mitochondrial function include higher generation of $O_2^{\bullet -}$, which may affect supercomplexes by increasing oxidative damage of IMM lipids and proteins, and diminished bioenergetic reserve capacity. RCF1a and RCF1b: human homologues of the yeast respiratory supercomplex factor (Rcf1). HIG2A: hypoxia inducible gene 1, domain family member 2A.

3.5 Supercomplex Destabilization and Impaired Mitochondrial Bioenergetics in Aging

Since their elucidation by Schägger and colleagues [118, 159, 213], it has been hypothesized that the biological reason for the existence of supercomplexes is to efficiently pass electrons through the ETC to O_2. Conversely, age-associated decrements in supercomplex assembly may theoretically result in inefficient ETC electron flux, adversely affecting energy reserve capacity. Experimental support for a role of supercomplex assembly in efficient electron transport comes from studies showing stability of the I_1III_2 supercomplex correlates with higher NADH-cytochrome *c* oxidoreductase activity [123, 124, 126]. Moreover, pioneering work by Zhang et al. [214] showed that supercomplex destabilization in a yeast strain lacking the cardiolipin synthase gene (i.e. *crd1*Δ mutants) was associated with abnormal growth on non-fermentable substrates [214]. In addition, Greenberg et al. showed that *crd1*Δ yeast displayed defective mitochondrial respiration and oxidative phosphorylation characteristics but only under extreme conditions, such as elevated temperature or osmotic shock [227-229]. Thus, it appears that supercomplexes are not required for maintaining basal respiratory activity

but for supporting bioenergetic reserve capacity. Taken together, these observations provide support that supercomplex destabilization diminishes State 3 respiration and also limits bioenergetic reserve capacity *in vivo*. This phenotype parallels characteristics of mitochondrial decay and at least in part explains the metabolic limitations of aging in aerobically active tissues such as the heart (see Section 1.1). Significantly more work will be needed in order to fully elucidate the true implications of supercomplex destabilization on the loss of mitochondrial bioenergetics.

3.6 Supercomplex Destabilization and ROS Generation in Aging

An obvious advantage for the ETC integrated as a supercomplex would be for efficient electron flux from reduced coenzymes to molecular oxygen [118]. In this regard, Panov et al. hypothesized that supercomplexes prevent superoxide formation even during maximal electron flux by maintaining all the electron carriers (i.e. Fe-S clusters and ubiquinone in complexes I and III) involved in $O_2^{\bullet-}$ generation in a permanently oxidized state [230]. Thus, supercomplexes would represent an evolutionary adaptation to prevent excessive ROS formation [230]. Alternatively, lack of supercomplexes has been implicated in high basal mitochondrial $O_2^{\bullet-}$

production in human neutrophils [148]. One study showed that mitochondria from mononuclear leukocytes and human leukemia cells (HL-60) contain $I_1III_2IV_N$ supercomplexes, but these particular macromolecular assemblies disappear when HL-60 cells differentiate into neutrophils [148]. While the respiratory supercomplexes were not necessary to maintain membrane potential ($\Delta\psi_m$), supercomplex loss in differentiated cells resulted in significantly higher rates of $O_2^{\bullet-}$ generation [148]. Furthermore, Lenaz et al. showed that mouse fibroblasts expressing the activated form of the *k-ras* oncogene lacked supercomplexes, which correlated with a higher rate of ROS appearance with respect to wild type fibroblasts [124]. Finally, new insights as to the role of supercomplexes in preventing ROS formation have been provided by the recent discovery of the Rcf1 subunit in yeast mitochondria [145, 225]. In these studies, *rcf1*Δ yeast displayed significantly higher rates of ROS appearance and increased oxidative damage versus wild type cells [145, 225].

In summary, supercomplex disassembly in aged post-mitotic tissues correlates with a wealth of information showing higher superoxide leak from the ETC with age. Taken together with the aforementioned studies on supercomplexes and their influence on $O_2^{\bullet-}$ generation, it is enticing to speculate a cause-and-effect relationship between higher ROS and

supercomplex deterioration. Figure 3.3 shows a schematic representation of this hypothesis. Nevertheless, there is still a dearth of experimental support for a functional role of supercomplexes in preventing higher rates of ROS appearance in post-mitotic tissues.

3.7 Concluding Remarks

Age-related deterioration of mitochondrial supercomplexes may be multifactorial where both IMM lipids (e.g. cardiolipin) and proteins may be involved. The precise elucidation of the factor(s) that integrate ETC components as supercomplexes should also shed light on age-related decline in supercomplex assembly and, by analogy, provide molecular target(s) for therapeutic intervention to maintain ETC function. In Chapter 4, we present an initial characterization of a potential correlation between age-associated alterations in those IMM constituents and supercomplex deterioration in cardiac mitochondria.

In addition, equal efforts should be directed toward defining the consequences of supercomplex disassembly with respect to known characteristics of mitochondrial decay. It should be noted that even small losses of supercomplex levels correlate with severe consequences to organ

function (*cf* Barth syndrome). However, studies with yeast suggest that basal bioenergetics is not affected with supercomplex disassembly. It is thus likely that supercomplex deterioration would mainly limit electron transport efficiency, leading to enhanced ROS/oxidative damage, as well as limitations in energy reserve capacity. Nevertheless, further studies are needed to elucidate whether defective supercomplex organization limits energy supply and aerobic metabolism in the aging animal.

Chapter 4

The Age-Related Loss of Cardiolipin May Be an Important Cause of Supercomplex Destabilization in Rat Heart Mitochondria

Luis A. Gómez and Tory M. Hagen

4.1 Abstract

In Chapter 2, we showed that aging adversely affects supercomplex organization in rat heart interfibrillar mitochondria (IFM). Nevertheless, the key factor(s) that regulate supercomplex stability in the myocardium are not fully understood. Moreover, it is not clear whether supercomplex deterioration only affects IFM or the two mitochondrial populations that exist in the heart display similar supercomplex deficits with age. In this chapter, we present an initial characterization of potential cause(s) of supercomplex deterioration in the aging heart. To this end, IFM and subsarcolemmal mitochondria (SSM) were isolated from young (4 months) and old (24 months) Fischer 344 rats. In agreement with results in Chapter 2, supercomplexes comprised of various stoichiometries of complexes I, III and IV, which typically organize as high mass (1500-2300 kDa) assemblies, were found in IFM and SSM. In contrast to IFM, where assemblies with the highest molecular weights (i.e. 1900-2300 kDa) were primarily destabilized with age, deficits in SSM supercomplexes were mainly confined to assemblies comprised of only one copy of complex IV ($I_1III_2IV_1$, 1700 kDa) (37% loss; p = 0.06). Technical limitations due to limited purified supercomplex material prevented an MS/MS elucidation of potential

changes to protein factors that might be involved in the age-related destabilization of supercomplexes. Nevertheless, the content of $(18:2)_4$-cardiolipin, the predominant species in the heart, was found to decline by 50% ($p < 0.05$) on average in both populations of cardiac mitochondria. Thus, the data presented in this chapter indicate that changes in cardiolipin may be at least one of the factors involved in supercomplex destabilization in the aging heart.

Keywords: Aging; cardiac mitochondria; supercomplexes; cardiolipin

4.2 Introduction

Two subpopulations of mitochondria with different subcellular locations as well as distinct functional features are present in cardiac tissue [209, 231]. Subsarcolemmal mitochondria (SSM) are located underneath the sarcolemma and are mainly associated to energy production for cellular ion exchange and homeostasis [232, 233]. Interfibrillar mitochondria (IFM) are intercalated along the myofibrils, and appear to supply ATP for the myocardial contraction-relaxation cycle [232, 234]. Interestingly, different studies show that deterioration of mitochondrial bioenergetics is only evident in IFM while SSM function stays unaffected in the aging rat heart [23, 25, 26, 54]. Nevertheless, it is not currently known, to our knowledge, whether age-related alterations in supercomplexes are also part of such asymmetrical decline in cardiac mitochondrial function. This characterization is important as understanding how aging affects supercomplex organization in the two mitochondrial subpopulations could contribute to elucidate the underlying factors(s) of supercomplex stability in the rat heart. Moreover, understanding such molecular factors of supercomplex destabilization in cardiac mitochondria could also contribute

to elucidate the mechanism(s) of the age-related deterioration of supercomplexes observed in other tissues such as the brain [154].

Although the underlying factor(s) of supercomplex deterioration are not fully known, the hypothesis that age-related alterations of the IMM constituents (e.g. ETC proteins and/or lipids) may be important factors of supercomplex destabilization in cardiac mitochondria was investigated in this chapter. In this respect, recent studies in yeast show that a newly identified mitochondrial protein, Rcf1 (respiratory supercomplex factor 1, which is homologous to the *HIG2A* gene product in mammals), associates with complex IV and plays a key role in supercomplex stabilization [145, 225, 226]. In addition, Rosca et al. reported a direct correlation between changes in phosphorylation of complex IV and alterations of supercomplex stability in a canine model of acute heart failure [138, 144].

On the other hand, accumulated evidence indicates that the mitochondrial phospholipid cardiolipin is also required for stabilization of ETC supercomplexes. Particularly, a correlation between severe supercomplex deterioration and alterations in cardiolipin has been observed in the *crd1*Δ yeast, a strain that lacks the cardiolipin synthase gene [214, 215]. Also, supercomplex deficits have been observed in lymphoblasts isolated from Barth syndrome patients [149], where severe alterations in

cardiolipin are caused by a mutation of the tafazzin gene (*TAZ*), which encodes the main enzyme involved in cardiolipin remodeling [235-238]. Therefore, it is plausible to hypothesize that proper cardiolipin acyl-chain composition rather than global levels of this phospholipid may be an important regulator of supercomplex stability. This hypothesis is supported by the study conducted by van Gestel et al. where alterations in molecular species of cardiolipin markedly diminished supercomplex stability in mitochondria from the yeast lacking the tafazzin gene (*taz1*Δ mutants) [239].

In this chapter, a characterization of age-associated alterations of supercomplex stability was performed in two different subpopulations of rat heart mitochondria. In addition, liquid chromatography-tandem mass spectrometry (LC-MS/MS) characterization of supercomplex proteins and HPLC analysis of cardiolipin were used as approaches to elucidate potential factor(s) of supercomplex destabilization in the aging heart. Our data indicate that age-related changes in cardiolipin may be at least one of the factors involved in supercomplex destabilization in cardiac mitochondria. In addition, our data suggest that differences in supercomplex deficits may partially explain the age-related loss of respiratory capacity that apparently affects IFM but not SSM.

4.3 Materials and Methods

4.3.1 Materials

Sucrose, D-mannitol, MOPS, HEPES, subtilisin A, Trizma, ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), KCl and other salts were from Sigma-Aldrich (St. Louis, MO). 6-Aminohexanoic acid was purchased from Acros-Organics (Morris Plains, NJ). All chemicals were reagent grade or the highest purity obtainable. 1-Pyrenyldiazomethane (PDMA) was obtained from Invitrogen (Carsbad, CA). High purity digitonin and n-dodecyl-β-D-maltoside (DDM) were from Calbiochem (San Diego, CA). Bovine serum albumin (fraction V, fatty acid-free) was from EMD Chemicals Inc. (San Diego, CA). Bovine heart cardiolipin and tetraoleoyl-cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI).

4.3.2 Animals

Young (4 months) and old (24 months) male Fischer 344 rats were obtained from the National Institute on Aging animal colonies. The animals

were housed in approved facilities at Oregon State University and maintained by the Laboratory Animal Resources Center. All procedures for animal handling were in keeping with approved institutional animal care and use guidelines.

4.3.3 Isolation of cardiac mitochondria

Cardiac subsarcolemmal and interfibrillar mitochondria were isolated from both young and old rats as previously described [209], using subtilisin A to release mitochondria from the myofibrils [132]. All steps of the isolation were performed on ice or at 4ºC. Protein content was determined by the Lowry method using bovine serum albumin as the standard (total protein kit from Sigma-Aldrich, St. Louis, MO).

4.3.4 Extraction of mitochondrial lipids and analysis of cardiolipin molecular species by HPLC

Lipids were extracted from isolated rat hear mitochondria using 2-propanol:hexane (2:3; v/v), according to the method described by Kolarovic and Fournier [240]. HPLC analysis of cardiolipin molecular species was performed by using 1-pyrenyldiazomethane as a derivatizing agent, according to the method described by Ritov et al. [139]. 1-pyrenylmethyl

ester derivatives of cardiolipin were separated by isocratic HPLC on a 2690 Separations Module (Waters, Milford, MA), using a mobile phase composed of absolute ethanol and 0.5 mM H_3PO_4, flow rate: 1.0 ml/min, and a Discovery® C18 column (5 μm, 4.6 mm i.d. x 15 cm, Supelco, Bellefonte, PA). Cardiolipin derivatives were monitored by fluorescence (λexcitation= 340 nm, λemission= 395 nm) using a 2475 Fluorescence Detector (Waters, Milford, MA). Cardiolipin species were identified using bovine heart cardiolipin as standard(s) (Avanti Polar Lipids Inc., Alabaster, AL), and peak areas quantified from calibration curves for the corresponding standards. Results were normalized to protein content.

4.3.5 Analysis of electron transport supercomplexes by BN-PAGE

ETC supercomplexes were separated using one-dimensional BN-PAGE as previously described [132]. Solubilization of mitochondrial membranes was achieved using a digitonin-to-protein ratio of 8:1 (w/w). Proteins (100 μg aliquots per lane) were separated on NativePAGE Novex 3-12% Bis-Tris gradient gels (Invitrogen, Carsbad, CA). Protein bands were visualized with Bio-Safe Coomassie-G250 (BioRad, Hercules, CA), and gel images digitized (Alpha Innotech, San Leandro, CA). Densitometric in-gel

analysis of protein bands was performed using ImageJ (National Institutes of Health).

4.3.6 Proteomics analysis of ETC supercomplexes by LC electrospray ionization tandem mass spectrometry (ESI-MS/MS)

Following 1D BN-PAGE (150 μg protein per lane), proteins from mitochondrial supercomplexes were subjected to in-gel tryptic digestion, according to an adapted protocol from the method described by Fandiño et al. [212]. Tryptic peptides were trapped on a Michrom Peptide CapTrap column and a C18 column (5 μm, 0.3 mm i.d. x 25 cm, Agilent Zorbax 300SB-C18). A binary solvent system consisting of 2% aqueous acetonitrile with 0.1% formic acid (solvent A), and acetonitrile with 0.1 % formic acid (solvent B) was used. Peptides were trapped and washed with 1% solvent B for 3min. Peptide separation was achieved using a linear gradient from 3% B to 30% B at a flow rate of 4.0 μl/min over 35 minutes. LC-MS/MS analysis was conducted on a Thermo LTQ-FT MS instrument coupled to a Waters nanoAcquity UPLC system. The LTQ-FT mass spectrometer was operated using data-dependent MS/MS acquisition with a MS precursor ion scan, performed in the ICR cell, from 350-2000 m/z with the resolving power set to 100,000 at m/z 400, and MS/MS scans performed by the linear

ion trap on the five most abundant doubly or triply charged precursor ions detected in the MS scan.

Thermo RAW data files were processed with Proteome Discoverer v1.3.0. For database searching, Mascot (v2.3) was used to search the SwissProt database using the following parameters: the digestion enzyme was set to Trypsin/P and two missed cleavage sites were allowed. The precursor ion mass tolerance was set to 10 ppm, while fragment ion tolerance of 0.8 Da was used. The following dynamic modifications were considered: Cys carbamidomethylation (+57.02 Da), Met oxidation (+15.99 Da), phosphorylation of Ser, Thr and Tyr residues (+97.98 Da), Lys ubiquitination (+114.04 Da), 4-hydroxynonenal (HNE) adducts of Cys, His and Tyr (+156.12), Cys nitrosylation (+28.99), Tyr nitration (+44.99), and deamidation of Asn and Gln residues. Scaffold (v3.3.1) (Proteome Software, Portland, OR) was used for search data compilation and data evaluation with embedded X!Tandem database searching algorithm.

4.3.7 Statistical analysis

Data analysis for two-group comparisons was performed using a two-sided Student's t-test. Differences were considered statistically significant at $p < 0.05$. GraphPad Prism 5 was used for all analyses.

4.4 Results

4.4.1 Two subpopulations of mitochondria display different degrees of supercomplex destabilization in the aging rat heart

Following BN-PAGE analysis of digitonin-solubilized mitochondrial membranes, it was observed that SSM also display the pattern of protein bands that we previously characterized as ETC supercomplexes in rat heart IFM (Chapter 2 and [132]) (Figs. 4.1A and 4.2A). In particular, four high-mass (1700-2300 kDa) protein assemblies (S_1-S_4) previously identified as $I_1III_2IV_N$-type supercomplexes as well as the I_1III_2 supercomplex (S_C, 1500 kDa) were observed in SSM and IFM. Moreover, the same pattern of solubilized proteins was maintained in both populations of cardiac mitochondria from old animals (Figs. 4.1A and 4.2A).

As shown in Figure 4.1 (B and C), the aging defects of SSM supercomplexes were apparently confined to the assembly comprised of only one copy of complex IV (S_1, 1700 kDa) (37% loss; $p = 0.06$), while the higher molecular weight supercomplex sub-types (S_2-S_4) were not significantly altered with age. In agreement with results presented in

Chapter 2, analysis of IFM proteins showed that, in general, supercomplex levels declined by 12% ($p < 0.05$) with age (Fig. 4.2B); however, different degrees of supercomplex deterioration were observed, depending on the particular supercomplex investigated (Fig. 4.2C). Specifically, supercomplexes S_2, S_3 and S_4 declined by 13% ($p < 0.05$), 30% ($p < 0.05$) and 45% ($p < 0.05$) in IFM from old rats, relative to the young controls. Thus, mitochondria from distinct subcellular locations show different age-related degrees of supercomplex destabilization in the rat heart. Moreover, IFM appear to display the highest degree of supercomplex deterioration, where the high-mass assemblies (1900-2300 kDa) are the most adversely affected.

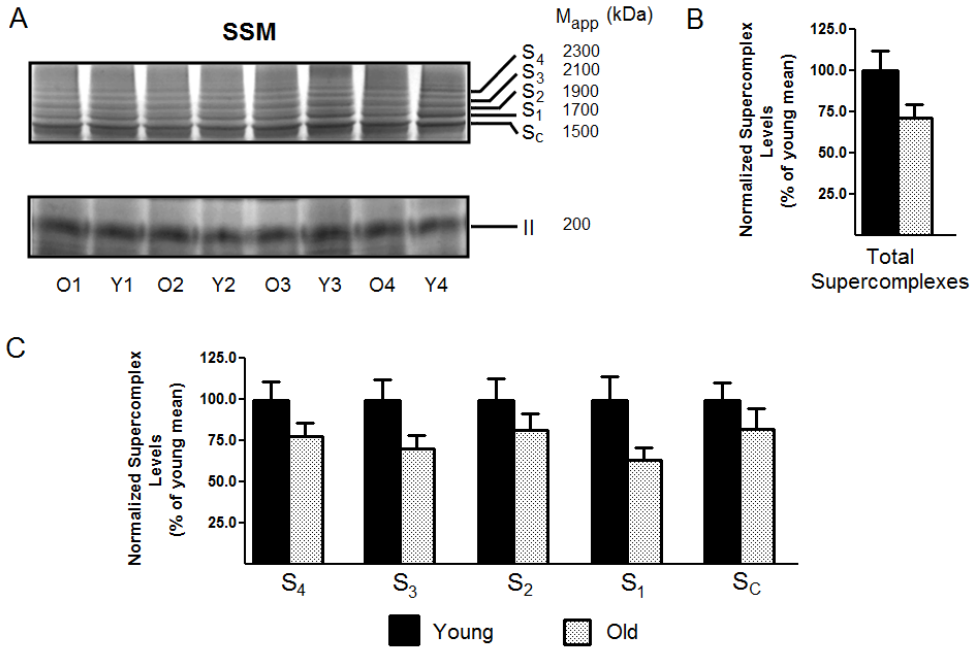

Figure 4.1 Age-related destabilization of ETC supercomplexes in rat heart SSM. (A) Separation of SSM supercomplexes from young (Y: 4 months) and old (O: 24 months) Fischer 344 rats, following BN-PAGE analysis of digitonin-solubilized mitochondrial membranes. S_C, S_N (N = 1-4) and II denote the I_1III_2 supercomplex, $I_1III_2IV_N$-type supercomplexes, and complex II of the ETC, respectively. (B) Total levels of supercomplexes were estimated by summing the densities of the four bands corresponding to $I_1III_2IV_N$ – type supercomplexes. Protein band densities were normalized to the density of complex II in each lane. (C) Normalized levels of mitochondrial supercomplexes. Results are presented as the mean ± SEM, and plotted as a percentage of the mean from young controls.

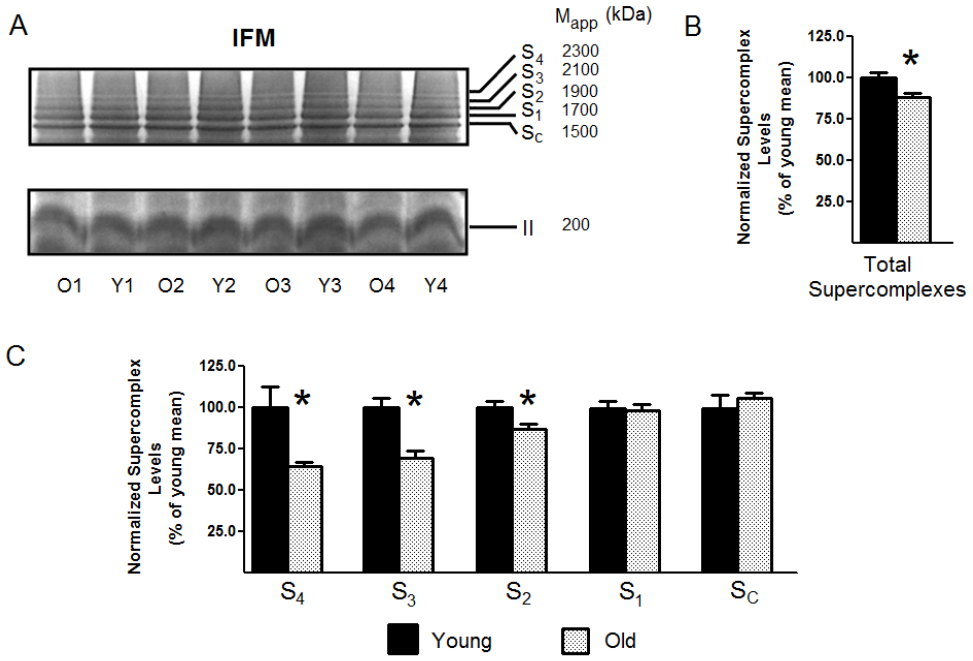

Figure 4.2 Age-related destabilization of ETC supercomplexes in rat heart IFM. (A) Separation of IFM supercomplexes from young (Y) and old (O) rats, following BN-PAGE analysis of digitonin-solubilized mitochondrial membranes. S_C, S_N (N = 1-4) and II denote the I_1III_2 supercomplex, $I_1III_2IV_N$ – type supercomplexes, and complex II of the ETC, respectively. (B) Total levels of supercomplexes were estimated by summing the densities of the four bands corresponding to $I_1III_2IV_N$-type supercomplexes. Protein band densities were normalized to the density of complex II in each lane. (C) Normalized levels of mitochondrial supercomplexes. Results are presented as the mean ± SEM, and plotted as a percentage of the mean from young controls. * $p < 0.05$ compared to young.

4.4.2 Aging results in lower levels of tetralinoleoyl-cardiolipin in two subpopulations of rat heart mitochondria

Age-related alterations in cardiolipin levels and/or composition were investigated as one of the factors that may affect supercomplex stability in rat heart mitochondria. As shown in Figure 4.3 (A and B), following HPLC analysis of mitochondrial lipid extracts (see Materials and Methods, Section 4.3.4), both SSM and IFM showed a similar profile of cardiolipin molecular species. In addition to $(18:2)_4$-cardiolipin, a symmetrical cardiolipin that was found to be the predominant species, $(18:2)_3(18:1)_1$-cardiolipin and $(18:2)_2(18:1)_2$-cardiolipin were also observed as minor constituents of cardiac mitochondria (Fig. 4.3, A and B). HPLC analysis of mitochondrial lipid extracts from old animals did not reveal significant alterations of the chromatographic profile of cardiolipin species with age (data not shown). However, the content of $(18:2)_4$-cardiolipin was found to decline by 50% on average in both populations of cardiac mitochondria (Fig. 4.3, C and D). Furthermore, analysis of the proportion of $(18:2)_2$-cardiolipin relative to $(18:2)_3(18:1)_1$-cardiolipin, an intrinsic indicator of the mitochondrial content of symmetrical cardiolipin [112], showed that aging significantly decreases molecular symmetry of cardiolipin in both SSM and IFM (Fig.

4.3, E and F). Interestingly, only a modest (10%, $p < 0.05$) loss in the global content of cardiolipin was found in IFM from old animals, relative to young controls (data not shown). Therefore, significant alterations in the content of symmetrical cardiolipin, namely $(18:2)_4$-cardiolipin, were found in two mitochondrial subpopulations in the aging rat heart, although total levels of cardiolipin only declined in IFM.

4.4.3 Potential factor(s) of supercomplex destabilization in the aging heart

As discussed in Chapter 3, new ETC proteins (e.g. Rcf1 in yeast, which is homologous to the *HIG2A* gene product in mammals) and/or certain mitochondrial phospholipids (e.g. cardiolipin) may play an important role in maintaining supercomplex stability. Using LC-MS/MS analysis, we sought to investigate potential changes to the Rcf1-type protein, other protein factors or protein oxidation that might be involved in supercomplex deterioration in cardiac aging. Particularly, we examined age-related differences in protein oxidative damage (i.e. HNE adducts, Tyr nitration, and Cys nitrosylation) and/or post-translational modifications (i.e. protein phosphorylation) of the ETC complexes assembled into supercomplexes. Also, we investigated whether ETC supercomplexes were

associated to specific mitochondrial factor(s) such as the Rcf1-type protein. Unfortunately, technical limitations due to limited purified supercomplex material prevented LC-MS/MS elucidation of potential changes to the Rcf1-type protein, other protein factors or protein oxidation that might be involved in the age-related destabilization of supercomplexes. Nevertheless, the strong parallel between supercomplex destabilization and changes in $(18:2)_4$-cardiolipin indicate that this alteration in cardiolipin is at least one of the factors that contribute to supercomplex deterioration in the aging heart. Table 4.1 shows a summary of the factors known to affect supercomplex organization in yeast and mammalian mitochondria, and their potential role on the age-associated destabilization of supercomplexes in cardiac mitochondria.

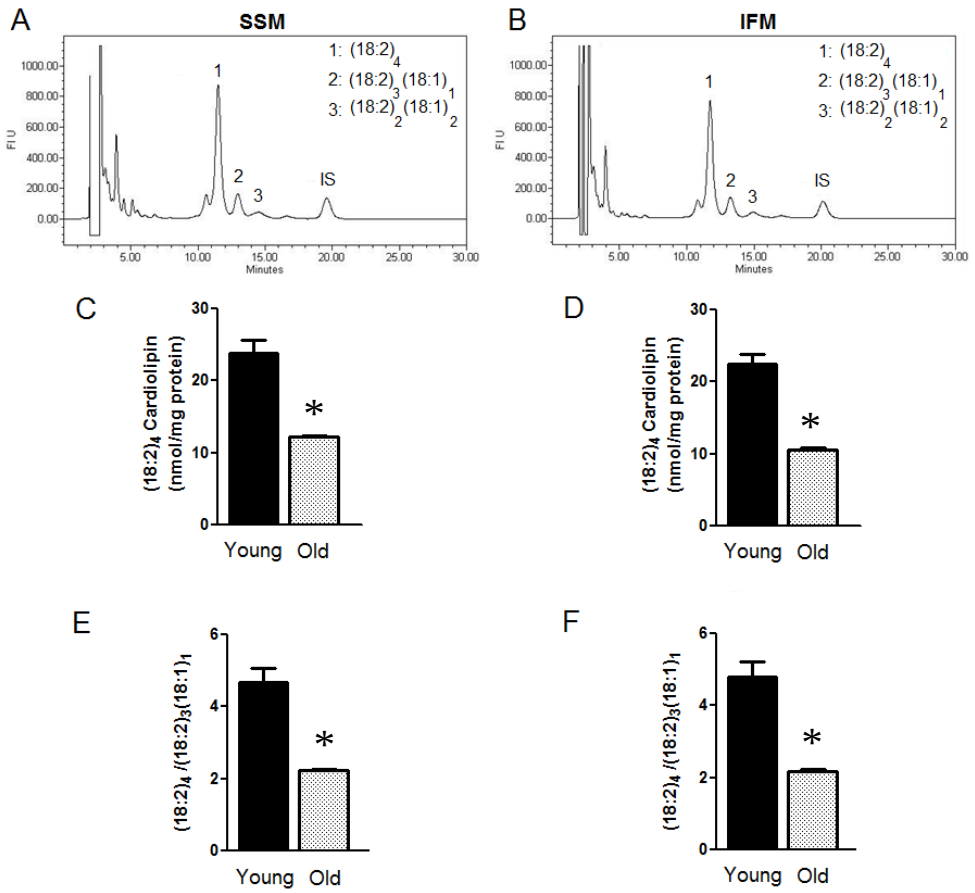

Figure 4.3 Tetralinoleoyl-cardiolipin declines with age in two subpopulations of rat heart mitochondria. Analysis of cardiolipin molecular species in SSM (A) and IFM (B) isolated from young (n=4) and old (n=4) rats. Aging decreases the predominant molecular species, tetralinoleoyl-cardiolipin [$(18:2)_4$-cardiolipin], in both SSM (C) and IFM (D). The ratio between $(18:2)_4$-cardiolipin and $(18:2)_3(18:1)_1$-cardiolipin was determined as an intrinsic indicator of the symmetrical cardiolipin content in SSM (E) and IFM (F). All results are presented as the mean ± SEM. *$p < 0.05$ compared to young animals. IS denotes the internal standard, $(18:1)_4$-cardiolipin.

Table 4.1 Potential factors involved in stabilization of mitochondrial supercomplexes

Mitochondrial factor	Role on supercomplex stability	Characterization in aging rat heart mitochondria
Loss of cardiolipin	Destabilization *crd1*Δ *yeast* [214, 215][a] *Human lymphoblasts* [149] *(Barth syndrome)*	Symmetrical cardiolipin decreases (see Fig. 4.3)
Rcf1protein	Stabilization *Yeast* [145, 225, 226] *C2C12 cells* [145]	n.d.[b]
Modifications of ETC proteins		
Posttranslational	Destabilization Phosphorylation of CIV [138] *(Dog heart mitochondria)*	n.d.
Oxidative damage	Destabilization Higher ROS generation [224] *(Mouse lung fibroblasts)*	n.d.

[a] Numbers in brackets denote references
[b] n.d. = not detected by LC-MS/MS analysis of supercomplex protein bands resolved by BN-PAGE.

4.5 Discussion

Alterations of the macromolecular organization of the ETC complexes I, III and IV have been characterized as part of the etiology of several pathologies associated with severe mitochondrial dysfunction such as Barth syndrome [149], Leigh syndrome [134, 135] and heart failure [144]. Moreover, some studies, including our own (Chapter 2 and [132]), now suggest that supercomplex destabilization could also be an intrinsic factor of the mitochondrial decay that affects highly aerobic tissues during the aging process [132, 154, 220]. Nevertheless, to the best of our knowledge, a full characterization of the underlying factor(s) of supercomplex deterioration in aged tissues is not currently available.

In agreement with our previous observations (Chapter 2 and [132]), age-related destabilization of ETC supercomplexes was observed in rat heart mitochondria. However, the data presented in this chapter provide a more detailed and complete characterization of such alterations as the effect of aging on supercomplex organization was examined in two different subpopulations of cardiac mitochondria. Our results indicate that both SSM and IFM display some degree of supercomplex destabilization with age. Nevertheless, IFM appear to exhibit higher supercomplex deficits than

SSM. The more robust supercomplex deficits noted for IFM fit well with previous observations that electron transport characteristics of this subpopulation are more adversely affected with age than SSM [23, 25]. Because assemblies comprising more than one copy of complex IV, which exhibit the most complex stoichiometry, underwent maximal deterioration with age in IFM, it is enticing to hypothesize that such supercomplex deficits may be a cause of impaired mitochondrial bioenergetics. Specifically, alterations in higher order supercomplexes could limit proper electron transfer to O_2 for its reduction [118, 119], selectively affecting mitochondrial respiratory capacity in IFM. In an extension of this hypothesis, the age-related increase in ROS appearance, which is more evident in IFM than SSM [26], could stem from higher superoxide formation due to altered supercomplex organization. Thus, our data suggest that age-related alterations in supercomplex organization are asymmetrical with respect to mitochondrial subcellular location in the rat heart.

Because potential changes to the Rcf1-type protein, other protein factors or protein oxidation that might be involved in the age-related destabilization of supercomplexes could not be discerned in this work, it is still unclear whether such alterations affect supercomplex organization in the aged heart. As we showed in Chapter 3, differences in supercomplex

organization are evident even in mitochondria from highly homologous sources (Rhesus monkey heart vs. Fischer 344 rat heart). Therefore, there is a rationale that supercomplex organization and stability involves different components of the IMM rather than being limited to changes in cardiolipin. In this respect, expression of the *HIG2A* and *HIG1A* genes, which is much higher in heart and brain than in liver [241], may explain the total absence of $I_1III_2IV_N$ supercomplexes in this organ [130] whereas those assemblies are a characteristic feature of mitochondria from heart and brain tissue [130]. It is also interesting that such a specificity of supercomplex organization does not appear to be only explained by differences in cardiolipin (see below) as $(18:2)_4$-cardiolipin represents more than 50% of the total cardiolipin pool in liver (Table 1.1). As analyzed in Table 4.1, post-translational modifications of the ETC complexes [138] and/or the presence of new accessory proteins (i.e. Rcf1-type protein) [145, 225, 226] may be important regulators of supercomplex organization in the aging rat heart.

On the other hand, cumulative evidence now indicates that the mitochondrial phospholipid cardiolipin is one of the factors involved in stabilization of supercomplexes [149, 215, 221]. For instance, severe supercomplex deterioration has been observed in the *crd1*Δ yeast [214,

215]. Moreover, using metabolic flux control analysis of intact mitochondria, Zhang et al. showed that cytochrome *c*-mediated electron transport between complexes III and IV in *crd1*Δ mutants follows a random-collision model rather than a solid state-type kinetics, which indicates loss of supercomplex organization in cardiolipin deficient mutants [242]. Finally, ETC supercomplexes have been reported to deteriorate in Barth syndrome, a condition where severe alterations in cardiolipin levels and composition are characteristic [149, 238, 243]. However, whether age-associated modifications of cardiolipin are an important factor of supercomplex destabilization is not fully understood. Furthermore, this analysis becomes more complex when investigating supercomplex organization in cardiac tissue due to the presence of two mitochondrial populations with different subcellular locations and distinct physiological function [209, 231]. In addition, discrepancies related to how aging affects cardiolipin levels and/or composition in cardiac mitochondria add complexity to this characterization.

Several laboratories have reported that cardiolipin declines ~30% on average in the aging rat heart [102, 103, 106, 107]. Nevertheless, Moghaddas et al. reported lack of age-related changes in cardiolipin levels in rat heart IFM [109]. The reason(s) for these discrepancies are not well

understood; however, it is reasonable to hypothesize that they are caused by differences in both the method(s) used for extraction of mitochondrial lipids and the technique(s) used for cardiolipin analysis. In this work, we decided to use a single-phase system that has been shown to provide higher efficiency for cardiolipin recovery during extraction of mitochondrial lipids [240] as a means to exclude artifactual alterations in cardiolipin levels due to differences in its extraction from mitochondria. Using this approach, our data show that an age-related loss (10%, $p < 0.05$) of cardiolipin parallels deterioration of supercomplexes in rat heart IFM. Interestingly, a significant loss of $(18:2)_4$-cardiolipin, the symmetrical cardiolipin species with the highest abundance in cardiac mitochondria [69], was observed in both mitochondrial subpopulations. Thus, it is not evident from our results whether age-associated alterations in $(18:2)_4$-cardiolipin also play a role in destabilization of supercomplexes. In addition, our data are in good agreement with a previous report by Lee et al. where a significant loss of linoleic acid (18:2n-6) was observed in the aging rat heart. Furthermore, our results partially agree with the study by Moghaddas et al. in that only a modest (10%, $p < 0.05$) age-related reduction in global levels of cardiolipin was found in IFM.

Because mitochondria from Barth syndrome patients display an abnormal profile of cardiolipin species, which is caused by a mutation of the tafazzin gene (*TAZ*) that encodes a mitochondrial transacylase enzyme involved in cardiolipin remodeling [235-238], it appears most likely that proper cardiolipin composition rather than its content is important to maintaining supercomplex organization. In this respect, using a proteomic characterization of supercomplexes in the *taz1*Δ yeast, van Gestel et al. showed that proper molecular cardiolipin is an important factor of supercomplex stability [239]. As cardiolipin is a molecule with a prochiral center, the central carbon atom of the main glycerol motif [69], it is plausible to hypothesize that the relatively high (~70-80%) content of symmetrical cardiolipin, namely $(18:2)_4$-cardiolipin, present in cardiac mitochondria across species [112, 137, 138, 152], is a unique means by which this lipid can mediate protein-protein interactions and maintain supercomplex organization. Thus, a symmetrical cardiolipin would have better fitting into asymmetrical binding site(s) on the ETC proteins comprised in supercomplex assembly. In fact, using electron paramagnetic resonance to characterize binding of spin-labeled lipids to membrane proteins in rat liver mitochondria, Schlame et al. [223] showed that the relatively high $(18:2)_4$-cardiolipin content (58.5%) confers special affinity

characteristics for cardiolipin binding to proteins. Taken together, the data presented here indicate that changes in cardiolipin may be at least one of the factors involved in supercomplex destabilization in the aging heart.

Chapter 5

Acetyl-L-Carnitine Supplementation Reverses the Age-Related Decline in Carnitine Palmitoyltransferase 1 (CPT1) Activity in Interfibrillar Mitochondria without Changing the L-Carnitine Content in the Rat Heart

Luis A. Gómez, Shi-Hua D. Heath, and Tory M. Hagen

Mechanisms of Ageing and Development
133 (2012) 99–106

Elsevier Inc. (Corporate Office)
360 Park Avenue South,
New York, NY 10010

5.1 Abstract

The aging heart displays a loss of bioenergetic reserve capacity partially mediated through lower fatty acid utilization. We investigated whether the age-related impairment of cardiac fatty acid catabolism occurs, at least partially, through diminished levels of L-carnitine, which would adversely affect carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme for fatty acyl-CoA uptake into mitochondria for β-oxidation. Old (24-28 mos) Fischer 344 rats were fed ± acetyl-L-carnitine (ALCAR; 1.5% [w/v]) for up to four weeks prior to sacrifice and isolation of cardiac interfibrillar (IFM) and subsarcolemmal (SSM) mitochondria. IFM displayed a 28% ($p < 0.05$) age-related loss of CPT1 activity, which correlated with a decline (41%, $p < 0.05$) in palmitoyl-CoA-driven state 3 respiration. Interestingly, SSM had preserved enzyme function and efficiently utilized palmitate. Analysis of IFM CPT1 kinetics showed both diminished V_{max} and K_m (60% and 49% respectively, $p < 0.05$) when palmitoyl-CoA was the substrate. However, no age-related changes in enzyme kinetics were evident with respect to L-carnitine. ALCAR supplementation restored CPT1 activity in heart IFM, but not apparently through remediation of L-carnitine levels. Rather, ALCAR influenced

enzyme activity over time, potentially by modulating conditions in the aging heart that ultimately affect palmitoyl-CoA binding and CPT1 kinetics.

Keywords: Carnitine palmitoyltransferase 1; kinetics; aging; interfibrillar mitochondria; acetyl-L-carnitine.

5.2 Introduction

Aging entails adaptations in energy metabolism to maintain cardiac pump function [44, 107]. For example, fatty acid oxidation, which is typically the primary oxidizable substrate for myocardial bioenergetics, declines with age in rodents [107, 168, 169] and also in humans [44]. While glucose oxidation appears to compensate this loss [44, 107], there is increasing evidence that such a shift in metabolism comes at a price, primarily in lower bioenergetic reserve capacity that limits response to heightened energy demands [170, 173, 244]. Moreover, as myocytes have limited means for exporting fatty acids in the form of triacylglyceride [171], lower fatty acid oxidation may shunt lipids into non-oxidizing metabolic pathways and/or lipid storage in the myocardium [170, 172, 173]. In its extreme, the age-associated decline in fatty acid-driven mitochondrial bioenergetics may thus initiate a form of myocardial lipotoxicity [174-176]. Therefore, it is important to understand the mechanism for lower fatty acid oxidation in the aging heart muscle.

While age-associated alterations in cardiac energy metabolism are undoubtedly multifactorial, several reports implicate carnitine palmitoyltransferase 1 (CPT1) as a key enzyme in the shift away from fatty

acid oxidation [107, 177, 178]. CPT1, the rate-controlling enzyme for overall fatty acid β-oxidation, catalyzes the condensation of acyl-CoA with L-carnitine to form acyl-carnitine esters, which are subsequently transported into mitochondria for further catabolism [179-181]. There is a consensus that CPT1 activity declines with age in heart and skeletal muscle [85, 107, 177, 182]. Moreover, down-regulation of CPT1 activity correlates with lipid accumulation and insulin resistance in rat skeletal muscle [182, 183]. Therefore, a plausible hypothesis is that lower CPT1activity is an underlying factor in the decline in fatty acid-supported myocardial bioenergetics.

Multiple lines of evidence now indicate that mitochondrial decay is a fundamental factor that leads to alterations in energy metabolism in the aged heart [62, 189, 208]. Nevertheless, the exact biochemical events that cause such an alteration in energetics are not completely understood. Furthermore, the existence of two cardiac mitochondrial subpopulations which display different functional features [209, 231] increases the complexity of a thorough characterization of the molecular events underlying the age-related deterioration of mitochondrial function. Histologically, subsarcolemmal mitochondria (SSM) are associated with the sarcolemma, and appear to provide energy for the regulation of myocellular

ion exchange and homeostasis [232, 233]. On the other hand, interfibrillar mitochondria (IFM) are intercalated along the myofibrils, and are believed to supply ATP for the myocardial contraction-relaxation cycle [232, 234]. A number of studies show that IFM functionally deteriorate with age. This subpopulation exclusively displays decreased ADP-stimulated respiration, altered electron transport chain (ETC) components, higher rates of oxidant appearance, and increased susceptibility to both permeability transition and damage during ischemia and reperfusion [23, 25, 26, 54, 245].

To our knowledge, no direct characterization as to how myocardial aging affects CPT1 activity and/or substrate utilization in the two mitochondrial subpopulations has been undertaken. Thus, it is not known whether the aging lesion for fatty oxidation lies in a particular mitochondrial subpopulation or more generally results from cardiac decrements in L-carnitine levels which decline with age in humans [185, 186] and also in rats [187, 188]. Thus, the age-related loss of myocardial carnitine levels may diminish overall CPT1 activity, and/or exacerbate enzyme catalytic dysfunction in a particular mitochondrial subpopulation. If carnitine levels indeed contribute to lower CPT1 activity, then it is equally possible that general CPT1-mediated fatty acid oxidation can be remediated by increasing myocardial L-carnitine content. In this regard, cardiac

mitochondrial bioenergetics in aged rats has been improved following dietary supplementation with the L-carnitine analogue, acetyl-L-carnitine (ALCAR) [86, 102, 103, 189].

In the present work, the effects of aging and ALCAR supplementation on the activity of CPT1 were investigated in rat heart SSM and IFM. Our data show that aging selectively decreases CPT1 activity in IFM by reducing enzyme catalytic efficiency for palmitoyl-CoA utilization without inducing significant alterations in the kinetic parameters for L-carnitine. These findings suggest that the decline in IFM CPT1 activity could be a key factor in the mechanism by which fatty acid utilization decreases, and as a consequence, induces lipid toxicity in the aging heart [174, 175].

5.3 Materials and Methods

5.3.1 Materials

Sucrose, D-mannitol, MOPS, HEPES, nagarse, palmitoyl-CoA lithium salt, Trizma, L- carnitine hydrochloride, adenosine 5′-diphosphate sodium salt, L-glutamic acid sodium salt, L-malic acid sodium salt, ethylene glycol tetraacetic acid (EGTA), KCl and other salts were from Sigma-

Aldrich (St. Louis, MO). All other chemicals were reagent grade or the highest purity obtainable. L-[*methyl*-^{3}H]-carnitine hydrochloride (specific activity 80.0 Ci/mmol) was supplied by Amersham Biosciences (Piscataway, NJ). Bovine serum albumin (fraction V, fatty acid-free) was from EMD Chemicals Inc. (San Diego, CA). ALCAR was a gift of Sigma Tau (Pomezia, Italy).

5.3.2 Animals

Young (3-4 months) and old (24-28 months) male Fischer 344 rats were obtained from the National Institute on Aging animal colonies. The animals were housed in approved facilities at Oregon State University and maintained by the Laboratory Animal Resources Center. All procedures including diets and animal handling were in keeping with approved institutional animal care and use guidelines.

5.3.3 ALCAR supplementation

Old rats were given a 1.5% (w/v; pH ~6) solution of ALCAR in their drinking water. Animals were maintained on standard chow diet and provided water *ad libitum* for one, two or four weeks before sacrifice and isolation of cardiac mitochondria. The average water consumption was ~32

ml/rat per day, corresponding to a daily average ALCAR intake ~1.1 g/kg body weight. ALCAR intake was not found to significantly change body weight in old rats with respect to animals receiving a control diet. At the end of the four week supplementation period, body weight was 391 ± 4 g for animals receiving ALCAR (n = 4) and 374 ± 32 g for old control rats (n = 4).

5.3.4 Isolation of cardiac mitochondria

Cardiac subsarcolemmal and interfibrillar mitochondria were isolated from both young and old rats using the method previously described by Palmer et al. [209]. Briefly, SSM were isolated by differential centrifugation from heart homogenates. Proteolytic treatment of the homogenate with Nagarse (3 mg/g tissue) was used to release IFM from myofibrils. All steps of the isolation were performed on ice or at 4ºC. Protein content was determined by the Lowry method using bovine serum albumin as the standard (total protein kit from Sigma-Aldrich, St. Louis, MO).

5.3.5 Mitochondrial oxygen consumption

Respiratory characteristics of mitochondria were monitored using a SYS-ISO2 dissolved oxygen meter coupled with an OXELP oxygen

electrode (World Precision Instruments, Sarasota, FL). Mitochondria were suspended in a buffer composed of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl and 5.0 mM KH_2PO_4, pH 7.2, supplemented with 0.1% (w/v) bovine serum albumin (fraction V, fatty acid-free). The rate of ADP-stimulated oxygen consumption (i.e. state 3 respiration) was registered using a mixture of 40 μM palmitoyl-CoA, 2 mM L-carnitine and 2.5 mM L-malate. State 3 was monitored as the oxygen consumption rate following the addition of 600 μM ADP. All experiments were performed at 30°C.

5.3.6 CPT1 activity

CPT1 enzymatic activity in isolated cardiac mitochondria was measured by monitoring the formation of the palmitoyl-ester of L-[*methyl*-^{3}H]-carnitine (specific activity 80.0 Ci/mmol), as previously described [246, 247]. CPT1 activity was sensitive to inhibition by malonyl-CoA (data not shown).

5.3.7 Myocardial levels of carnitine

Tissue content of both free L-carnitine and (medium- and long-chain) acyl-carnitine was determined using an enzymatic cycling method

with carnitine dehydrogenase, as previously described [248, 249]. Quantitation of total carnitine was performed by determining the content of free L-carnitine upon hydrolysis of acyl-carnitine esters by L-acetylcarnitine hydrolase. Acyl-carnitine levels were calculated as the difference between the content of total carnitine and free L-carnitine. Enzymes and the assay kit for analysis of total and free carnitine levels (Kainos Laboratories, Inc., Tokyo, Japan) were a gift of Dr. Hirohiko Kuramatsu (Osaka University, Japan).

5.3.8 Statistical analysis

For two-group comparisons, results were analyzed using a two-sided Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. For multi-group comparisons, results were analyzed using the ANOVA F-test, together with the Tukey-Kramer procedure, setting the overall family-wise confidence level at 95%. Linear regressions were obtained using GraphPad Prism 5 software (GraphPad Software Inc., USA). Additionally, hyperbolic kinetics of CPT1 was established by comparing data fitted to either Michaelis-Menten or Allosteric-Sigmoidal models, using GraphPad Prism 5.

5.4 Results

5.4.1 CPT1 activity selectively declines in interfibrillar mitochondria and impairs fatty acid-driven bioenergetics in the aged rat heart

In order to investigate how age affects fatty-acid supported bioenergetics in the two mitochondrial subpopulations, both fatty acyl-CoA-mediated oxygen consumption characteristics and CPT1 activity were monitored. As shown in Figure 5.1A, palmitoyl-CoA-supported state 3 respiration significantly declined (41%, $p < 0.05$) in IFM isolated from old versus young animals. However, no significant changes in mitochondrial respiration were observed with age in SSM (Fig. 5.1B). In concert with these results, there was no significant difference in CPT1 activity in SSM; however, we observed a significant 28% loss in CPT1 activity in the IFM fraction (Table 5.1).

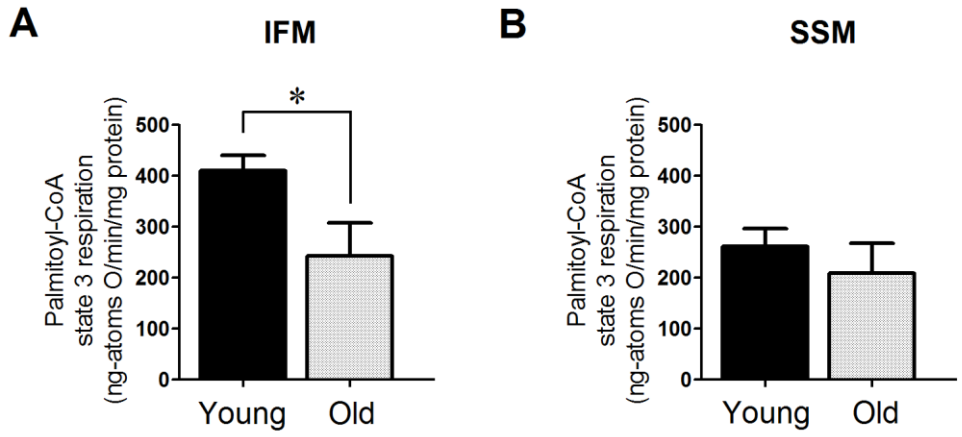

Figure 5.1 Fatty acid-driven respiration selectively declines with age in rat heart interfibrillar mitochondria. Palmitoyl-CoA-supported state 3 respiration was determined in two subpopulations of cardiac mitochondria, IFM (A) and SSM (B), isolated from young (n = 6) and old (n = 4) Fischer 344 rats. Mitochondrial oxygen consumption was measured as described in Materials and Methods (Section 5.3.5). All results are presented as the mean ± SEM. *$p < 0.05$ compared to young animals.

Table 5.1 CPT1 activity in two subpopulations of rat heart mitochondria.

Mitochondrial Subpopulation	CPT1 activity [a] (nmol/min/mg protein)	
	Young	Old
Subsarcolemmal mitochondria	7.33 ± 0.42	7.17 ± 0.46
Interfibrillar mitochondria	7.71 ± 0.43	5.59 ± 0.41[b]

Results are presented as the mean ± SEM. In all groups n = 7.
[a] Using a concentration of 70 μM palmitoyl-CoA and 400 μM L-carnitine in the assay mixture.
[b] $p < 0.05$ versus young IFM.

Because release of IFM from the myofibrils during the isolation process requires treating heart homogenates with a protease (see Materials and Methods, Section 5.3.4) that could differentially damage CPT1 in IFM, control experiments were performed to investigate whether the apparent decline in IFM CPT1 activity stemmed from an artifact of the isolation procedure or was a result of the aging process. Treatment of myofibrillar fractions with increasing concentrations of protease (i.e. from 0.3 mg/g tissue to 30 mg/g tissue) did not significantly affect CPT1 activity in IFM from old rat hearts. This indicates that CPT1 is not highly sensitive to the brief and relatively mild proteolytic treatment employed for IFM isolation. Moreover, further experiments where tissue homogenates were similarly treated with protease revealed no significant alterations of CPT1 activity in SSM of either young or old rat hearts (data not shown). Therefore, we conclude that the loss of CPT1 activity in IFM is a result of the aging process, and suggests that a differential decline in fatty acid-supported mitochondrial energy transduction occurs with age.

5.4.2 Aging alters CPT1 enzyme parameters in rat heart interfibrillar mitochondria

The CPT1 enzyme displays a complex mode of catalysis where overall catalytic activity can be affected by substrate affinity for either L-carnitine or fatty acyl-CoA. In order to discern a plausible mechanism as to the age-related decline in IFM CPT1 activity, kinetic parameters for both enzyme substrates were monitored. Lineweaver-Burk plots for palmitoyl-CoA utilization indicated that both V_{max} and K_m decrease with age (Fig. 5.2A), a kinetic shift which resembles uncompetitive inhibition. Further examination of the data using Eadie-Hofstee plots showed that V_{max} and K_m declined by 60% ($p < 0.05$) and 49% ($p < 0.05$), respectively (Fig. 5.2B). On the other hand, double-reciprocal plots with respect to L-carnitine did not indicate significant differences in CPT1 kinetic parameters in IFM from old relative to young animals (Fig. 5.2C). This observation was corroborated by Eadie-Hofstee analysis for both V_{max} and K_m (Fig. 5.2D). Taken together, these results show that the age-associated loss of CPT1 activity in IFM is mainly caused by a decrease in V_{max} for palmitoyl-CoA without a significant change in enzyme affinity for L-carnitine.

In order to elucidate how the age-associated changes in kinetic parameters with respect to palmitoyl-CoA could regulate the catalytic cycle

of IFM CPT1, the data were further analyzed using a theoretical model. Figure 5.3A shows that when a decrease in V_{max} predominates over an apparent increase in substrate affinity (i.e. lower K_m), the global result is a loss of catalytic efficiency. Moreover, this dominance is accentuated when substrate concentration is close to the K_m, which is most likely the case for catalysis under physiological conditions. Based on this theoretical construct, our data showing an age-related decrease of 60% and 49% in V_{max} and K_m (Fig. 5.2B), respectively, suggest that IFM CPT1 is 20% less catalytically efficient with age. This would result in a 45% decline in overall enzyme activity when the concentration of palmitoyl-CoA nears the K_m of the enzyme (i.e. 200 μM) (Fig. 5.3B). Thus, assuming that there is no age-dependent change in CPT1 levels (a reasonable assumption given no change in carnitine-supported kinetics), this analysis supports the concept that the palmitoyl-CoA-CPT1 complex is destabilized in IFM of aging rat hearts.

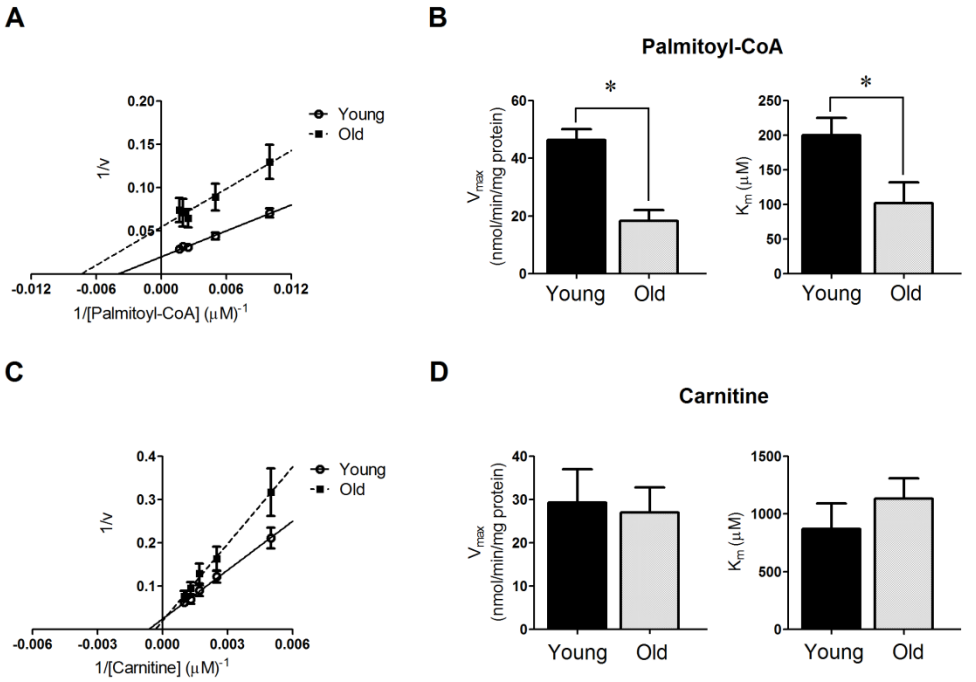

Figure 5.2 Age-associated changes in CPT1 enzyme kinetic parameters in rat heart interfibrillar mitochondria. CPT1 activity was measured in IFM as described in Materials and Methods (Section 5.3.6). For analysis of palmitoyl-CoA-mediated enzyme kinetics (A and B), CPT1 activity was measured using increasing concentrations of palmitoyl-CoA while maintaining L-carnitine at 400 μM (A and B). Conversely, the effect of increasing L-carnitine levels on CPT1 activity was measured in (C and D) where palmitoyl-CoA levels were kept constant at 70 μM palmitoyl-CoA. Panels (A) and (C) show double reciprocal plots with respect to palmitoyl-CoA and L-carnitine concentrations, respectively. For each concentration of substrate used, independent experiments were done using IFM from both young (n = 5) and old (n = 5) rats. Eadie-Hofstee plots of the raw data were generated with respect to palmitoyl-CoA (B) and L-carnitine (D) to assess both K_m and V_{max} for each substrate. Reciprocal velocity in (A) and (C) is expressed in (nmol/min/mg protein)$^{-1}$. All results are presented as the mean ± SEM. *$p < 0.05$ compared to young animals.

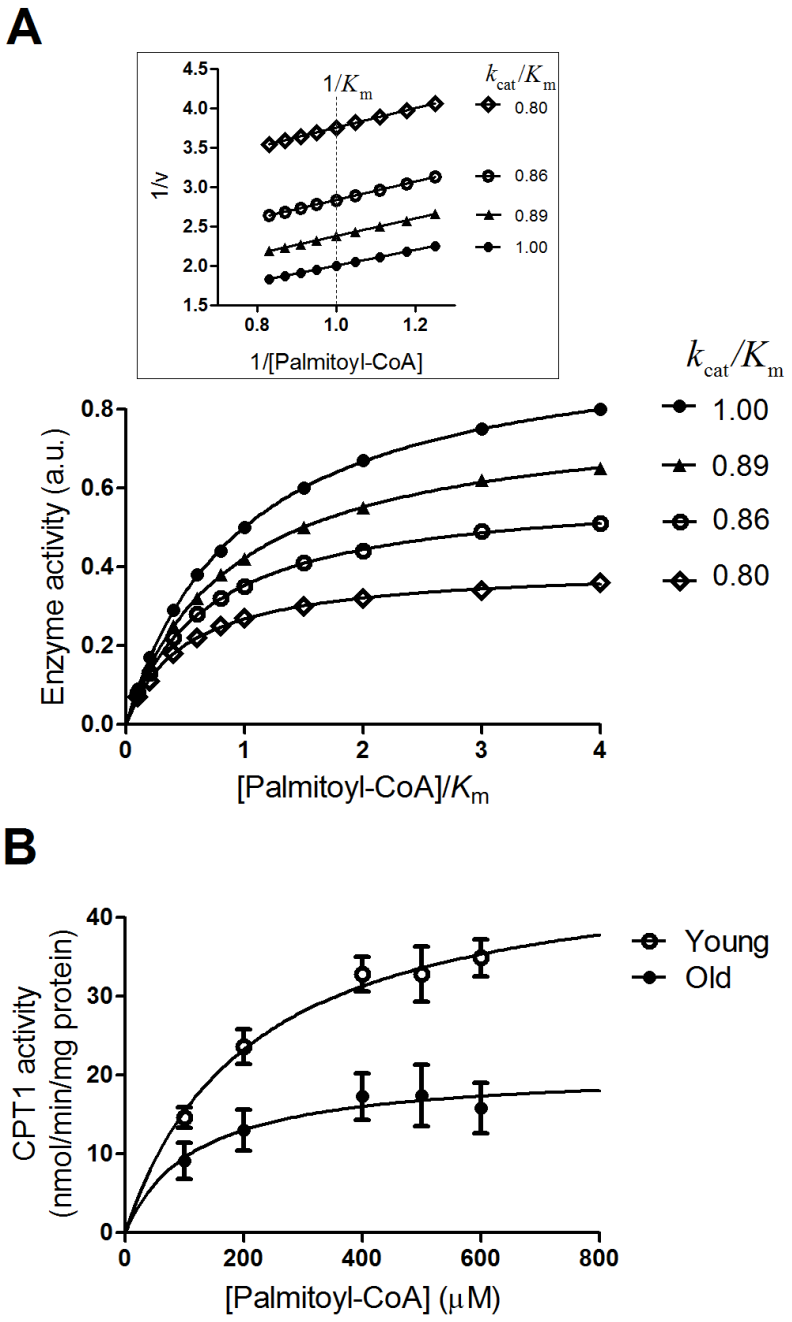

Figure 5.3 Theoretical analysis how CPT1 enzyme activity is modulated by fractional changes in V_{max}, K_m, and the turnover number (k_{cat}) for palmitoyl-CoA *(see legend on next page)*.

Figure 5.3 *(see previous page)* Theoretical analysis how CPT1 enzyme activity is modulated by fractional changes in V_{max}, K_m, and the turnover number (k_{cat}) for palmitoyl-CoA (see legend on next page)Enzyme rate (v) is expressed in terms of total enzyme [E_t] and substrate [S] concentration, as $v = \frac{k_{cat}[E_t][S]}{K_m + [S]}$ (A). This analysis was performed relative to the reference state where [E_t], V_{max}, K_m, and k_{cat}/K_m were set to 1.0 arbitrary unit (a.u.). A decrease in V_{max} when K_m also declines results in lower CPT1 catalytic efficiency when changes in V_{max} predominate over variations in K_m. Enzyme kinetics follows an uncompetitive inhibition-like behavior and is significant when [Palmitoyl-CoA]= $K_m \pm 0.2\ K_m$ (see also inset). Age-associated decline in CPT1 kinetics in rat heart IFM (B).

5.4.3 Carnitine levels decline with age and are partially restored by dietary supplementation with ALCAR

Because substrate limitations *per se* would adversely affect the formation of palmitoyl-carnitine at the organ level even in the absence of structural alterations of the IFM CPT1 enzyme, we also explored the possibility that lower fatty acid-supported bioenergetics was exacerbated by the age-related decline in myocardial levels of L-carnitine. To this end, both free carnitine and acyl-carnitine levels were measured in cardiac tissue from young and old animals. As shown in Figure 5.4, aging leads to a decrease in the content of myocardial carnitine. With respect to young rat hearts, levels of free carnitine (Fig. 5.4A), acyl-carnitine (Fig. 5.4B), and total carnitine (Fig. 5.4C) declined by 38% ($p < 0.05$), 56% ($p < 0.05$) and 42% ($p < 0.05$),

respectively, in old animals. Thus, fatty acid-mediated mitochondrial respiration may be attenuated by limited myocardial L-carnitine content.

As previous studies show that carnitine levels increase in cardiac tissue following ALCAR supplementation, we fed ALCAR to aged rats for up to four weeks prior to sacrifice and mitochondrial isolation. ALCAR supplementation failed to remediate the loss of either free- (Fig. 5.4A) or total-carnitine content (Fig. 5.4C) over a two-week supplementation period; however, acyl-carnitine levels were restored to those seen in young controls (Fig. 5.4B). Overall, ALCAR feeding established a new equilibrium in the cardiac acyl-carnitine/free carnitine ratio, resulting in a higher proportion of acyl-carnitine in the aging myocardium (Fig. 5.4C). These results suggest that CPT1 activity increased after ALCAR treatment, which is shown in Figure 5.5.

5.4.4 Feeding ALCAR to old rats reverses the age-related decline in CPT1 activity in interfibrillar mitochondria

As shown in Figure 5.5A, dietary supplementation of old rats with ALCAR increased IFM CPT1 activity over a four-week timecourse. Nevertheless, this increase was gradual and CPT1 activity did not advance to levels evident in young rats until relatively late in the timecourse (Fig.

5.5, A and C). On the other hand, ALCAR did not significantly affect CPT1 activity in SSM (Fig. 5.5, B and D). Thus, our results indicate that feeding ALCAR to old rats preserves fatty acid-supported mitochondrial bioenergetics by restoring the age-related decline in CPT1 activity in heart IFM. ALCAR does not appear to change substrate (i.e. L-carnitine) availability *per se* as the same dietary intervention had no significant effect on the reaction catalyzed by CPT1 in SSM.

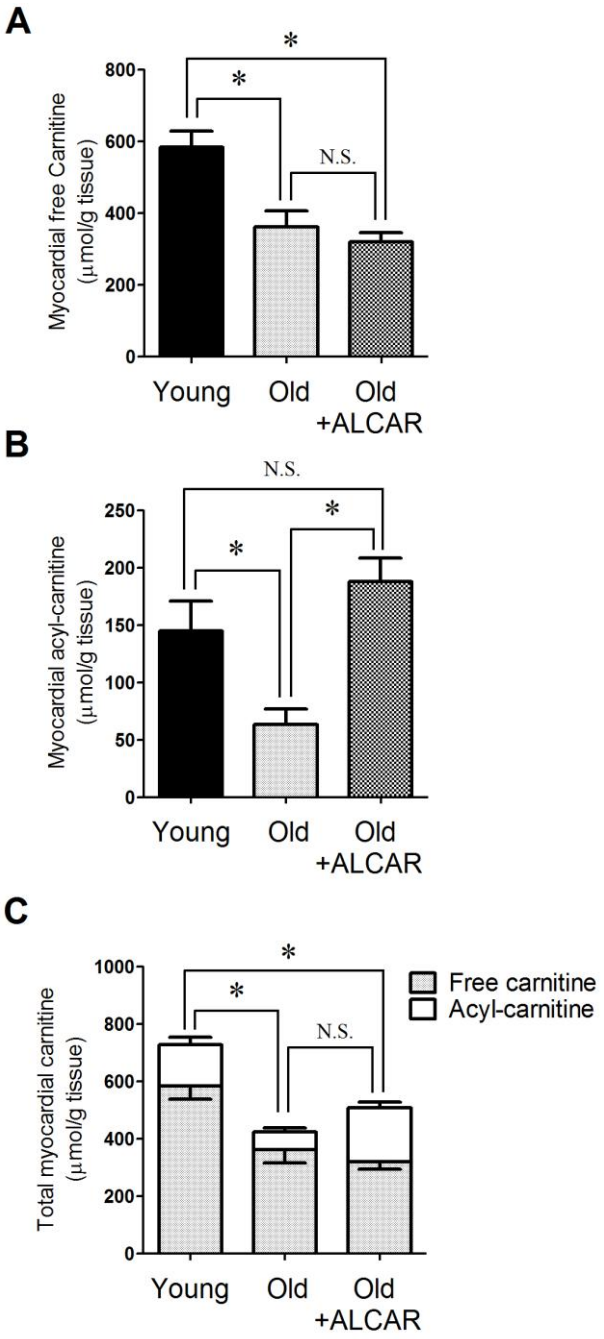

Figure 5.4 Myocardial carnitine levels decline with age but can be partially restored by dietary supplementation with acetyl-L-carnitine *(see legend on next page)*.

Figure 5.4 *(see previous page)* Myocardial content of free carnitine and acyl-carnitine was determined as described in Materials and Methods (Section 5.3.7). Free carnitine in cardiac tissue from young (n = 6), old (n = 5) and ALCAR-supplemented old rats (Old + ALCAR; n = 5) (A). Myocardial acyl-carnitine levels (B). Total carnitine content and its distribution in cardiac tissue (C). For (A) and (B), results are presented as the mean ± SEM. Error bars in (C) correspond to acyl-carnitine (upward) and free carnitine (downward). For simplification, error bars for total carnitine values are not shown. *$p < 0.05$ compared to young controls.

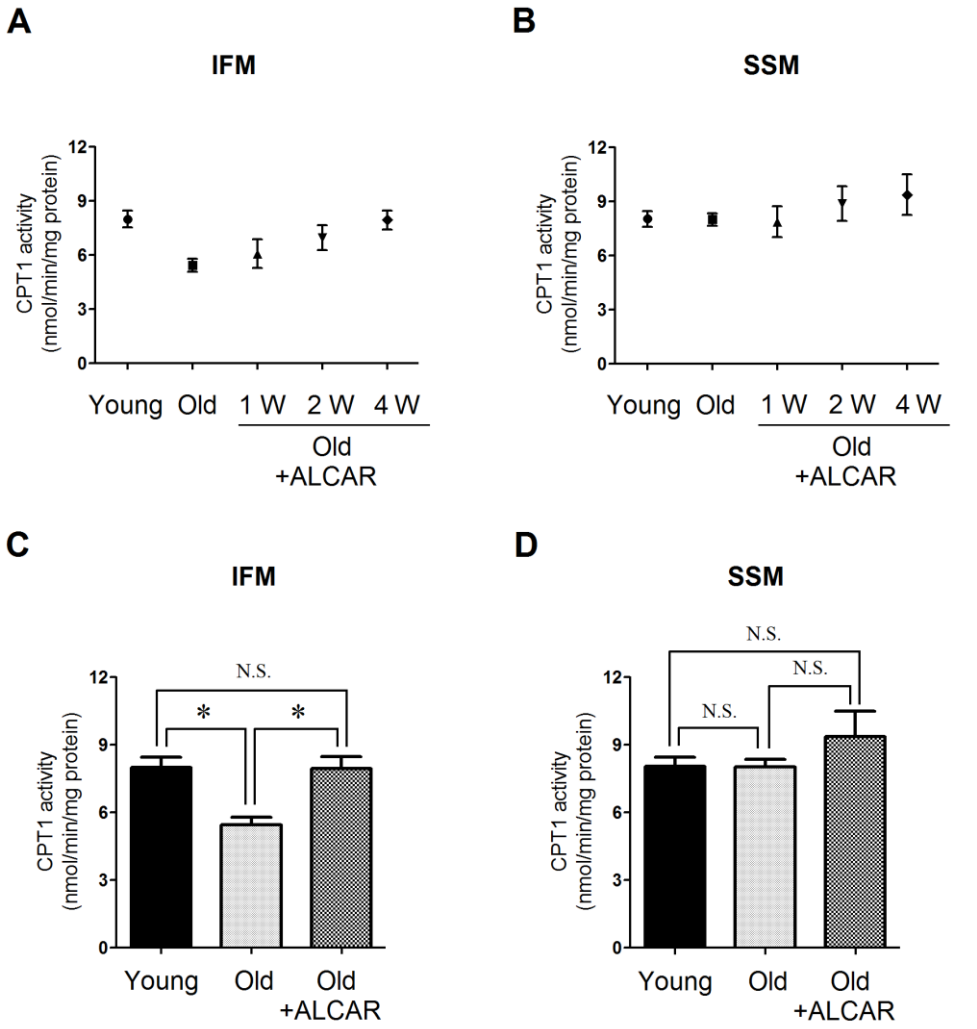

Figure 5.5 Dietary supplementation with acetyl-L-carnitine restores the age-related loss of CPT1 activity in rat heart interfibrillar mitochondria. CPT1 activity was measured using a concentration of 70 μM palmitoyl-CoA and 400 μM L-carnitine in the assay mixture, as described in Materials and Methods (Section 5.3.6). Enzyme activity in IFM (A and C) and SSM (B and D) isolated from young (n = 4), old (n = 4) and old + ALCAR (n = 4) rat hearts. Old animals were given ALCAR for one (1 W, n = 4), two (2 W, n = 4) or four (4 W, n = 4) weeks, as described in Materials and Methods (Section 2.3.3) (A and B). (C) and (D) correspond to four weeks of supplementation with ALCAR. All results are presented as the mean ± SEM. *$p < 0.05$ compared to young animals.

5.5 Discussion

The present work provides a new perspective on the age-associated decline in fatty acid-supported cardiac bioenergetics. To the best of our knowledge, this is the first side-by-side characterization of CPT1 activity in two sublocalized mitochondrial populations of the aging heart. Our data show that age induces a ~30% decline in CPT1 activity, but only in IFM (Table 5.1 and Fig. 5.5). While this activity loss is consistent with previous observations for diminished fatty acyl-CoA supported bioenergetics in hearts from old rats and mice [107, 168, 177], our results now indicate that the aging lesion for CPT1 is specific to mitochondria intercalated along the myofibrils. As the IFM supply ATP to the actomyosin complex during the contraction-relaxation cycle [232, 234], cardiac pump function may therefore be even more adversely affected with age than the subtle loss in overall CPT1 activity would generally indicate. These results thus have important implications for myocardial energy reserve capacity.

Based on both the observational and theoretical data generated in this study, it appears that the mechanism(s) underlying lower IFM CPT1 activity is complex. Modeling enzyme activity versus alterations in catalytic efficiency (Fig. 5.3A) suggests that the observed age-associated changes in

K_m and V_{max} for palmitoyl-CoA account for essentially all of the observed loss of CPT1 activity. Thus, even though there is a general age-dependent decrease in myocardial L-carnitine levels, its decline has little consequence to enzyme activity. These rather surprising results actually reinforce the concept that CPT1 activity loss is localized to a specific mitochondrial subpopulation as diminished L-carnitine content would adversely affect CPT1 activity in the SSM fraction as well. Considering that we also previously showed no age-associated alterations to malonyl-CoA levels in the aging rat heart [250], it now becomes clear that the age-specific loss of IFM CPT1 catalytic efficiency stems from modifications to the enzyme and is not because of alterations to substrate levels or allosteric effectors.

CPT1 follows a bi-bi ordered mode of catalysis where binding of a long-chain fatty acyl-CoA molecule to the enzyme active site initiates the reaction [179, 180]. Mutations of glycine residues in human hepatic CPT1 specifically affect the palmitoyl-CoA binding pocket and lead to loss of enzyme function [251, 252]. Furthermore, enzyme activity was completely abolished in mitochondria from yeast expressing the mutant liver-specific CPT1 protein where Gly^{710} was changed to a Glu residue in the protein hydrophobic core [251]. We therefore propose that the loss of catalytic efficiency for palmitoyl-CoA utilization stems from limitations in its

binding to the enzyme, thereby lowering overall catalytic activity even when L-carnitine levels are saturating. This type of altered enzyme kinetics is akin to an uncompetitive mode of inhibition where alterations of the enzyme substrate complex adversely affect overall catalytic efficiency of the enzyme (Fig. 5.6).

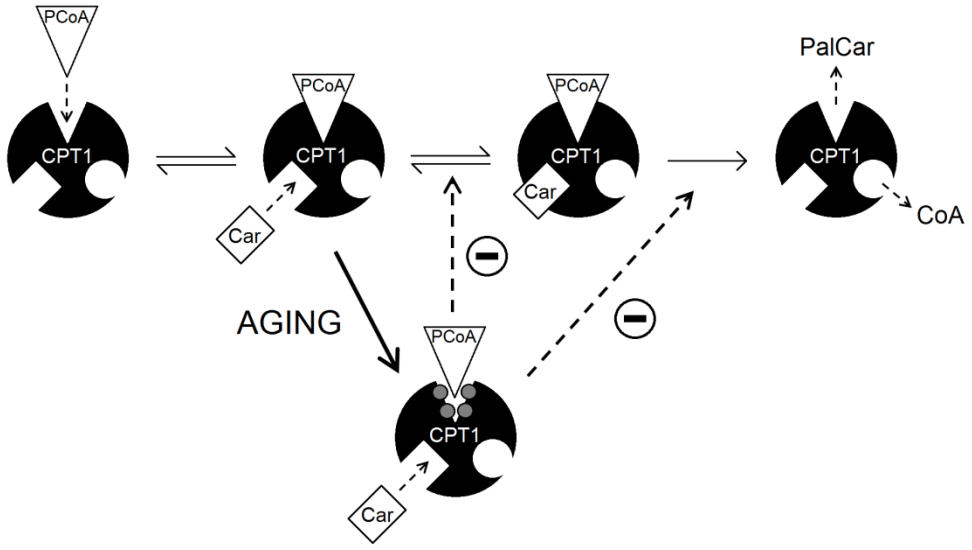

Figure 5.6 Schematic representation of the age-associated decline in CPT1 catalytic activity. Alterations of the CPT1-palmitoyl-CoA complex are hypothesized to act as an 'upstream' inhibitor of the formation of palmitoyl-L-carnitine. PCoA: palmitoyl-CoA; Car: L-carnitine; and CoA: coenzyme A.

The precise cause(s) leading to this uncompetitive mode of IFM CPT1 inhibition was not directly explored in the present study, and may be multifactorial. A plausible hypothesis is that age-associated oxidative modifications that selectively affect IFM CPT1 alter its catalytic activity by destabilization of palmitoyl-CoA, and that long-term supplementation of ALCAR reverses such alterations (see below). This concept is buttressed by previous reports showing that both CPT1 and carnitine octanoyltransferase contain specific binding sites for L-carnitine, acyl-CoA, and CoASH where amino acid residues in or proximal to the acyl-CoA binding region are key to stabilizing the acyl-CoA-enzyme complex during catalysis [252, 253]. Thus, reactive oxygen and nitrogen species, electrophiles, or small molecule conjugation, may alter critical amino acid residues at or near the palmitoyl-CoA binding pocket and limit formation of the initial enzyme-palmitoyl-CoA complex (Fig. 5.6).

While characterization of specific protein modification(s) that lead to the aging lesion in IFM CPT1 catalysis is beyond the scope of the present study, there is literature precedent suggesting that IFM are particularly susceptible to protein modification. First, we previously showed that aging leads to higher rates of IFM oxidant appearance versus SSM [26], which could promote oxidative modifications that selectively affect that

mitochondrial subpopulation. Second, Lesnefsky et al. [25] further reported that the cytochrome *c* binding site of complex III was specifically altered with age, but only in the IFM subpopulation. On the other hand, Sharma et al. [254] recently showed that incubating cardiac mitochondria with peroxynitrite ($ONOO^-$) initiated post-translational modifications of specific cysteine and tyrosine residues of CPT1, which in turn modulated enzyme activity. Taken together, these results suggest that aging causes an enhanced pro-oxidant milieu that is specific for IFM, thereby initiating conditions that adversely affect CPT1 catalysis in that mitochondrial subpopulation. We are currently examining the linkage between protein modification and the specific alterations to CPT1 activity seen in IFM of aging rat hearts.

We and others have previously found that supplementation of old rats with ALCAR remediates the age-related decay in mitochondrial bioenergetics in liver [94, 189], heart [86, 102], muscle [255] and brain [256]. ALCAR has been postulated to mediate this improvement in a straightforward manner, namely, by replenishing L-carnitine levels which otherwise decline with age [185, 187, 188, 257]. However, this report shows that even though ALCAR supplementation remediated overall IFM CPT1 activity loss, its mechanism(s) of action may be distinct from its essential role involving mitochondrial fatty acyl-CoA import. This concept

is consistent with our current results showing no role for L-carnitine on the age-associated decline in CPT catalytic efficiency, and also by the observation that IFM CPT1 catalysis improved slowly over the month-long ALCAR supplementation period. These results suggest a far more complex mechanism of action for this metabolite, which is not consistent with an immediate, direct replenishment of myocardial carnitine levels. Precisely how ALCAR improves IFM CPT1 activity in aged rat hearts is not currently known; however, a significant body of evidence indicates that it facilitates a number of metabolic changes [73, 94, 189, 255, 258], which may ultimately improve CPT1 activity specifically in IFM. Even though ALCAR is not a classical free radical terminating molecule, nevertheless, it decreases the formation of nitro-tyrosine protein adducts in alcohol-induced brain damage [259], and limits oxidative damage in the heart following ischemia/reperfusion injury [260, 261]. Also, proteomic analysis of aging rat brain shows that ALCAR lowers levels of protein carbonylation [262]. Furthermore, Gadaleta and coworkers revealed that the mitochondrial isoform of peroxiredoxin III was less oxidatively modified and more active in livers from old rats supplemented with ALCAR [73]. Additionally, ALCAR initiates increased mitochondrial biogenesis and turnover [255, 263], which would promote clearance of damaged IFM CPT1 proteins.

Finally, ALCAR rebalances membrane phospholipid content, and for mitochondria, reverses the age-related decline in cardiolipin levels, a key phospholipid necessary for proper electron transport chain function [52, 94, 103, 108]. Taken together, ALCAR may indirectly improve IFM CPT1 activity by maintaining protein integrity through membrane restructuring or limiting oxidative damage, which otherwise specifically increases in the IFM subpopulation with age. This hypothesis is supported by evidence that ALCAR increases cellular stress response, through the regulation of gene expression and the synthesis of important proteins that sustain the oxidative stress defense [264, 265]. In particular, ALCAR stimulates expression of heme oxygenase-1 (HO-1) and endothelial NO synthase (eNOS) in human endothelial cells during H_2O_2-induced oxidative stress [266]. Also, ALCAR decreases amyloid beta-mediated oxidative damage in primary cortical neuron cultures by again increasing expression of both HO-1 and heat shock protein 70 (Hsp70) [265]. Furthermore, in rats exposed to γ-radiation, ALCAR reverses the loss of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx), restores GSH levels, and prevents the accumulation of malondialdehyde (MDA) in the liver and the lungs [267]. Thus, it is conceivable that ALCAR improves IFM CPT1 activity by preventing oxidative damage to the protein.

Our current data warrants more detailed studies to discern the mechanisms involved in ALCAR-dependent remediation of CPT1 activity. Regardless of the precise nature of its action, however, it is clear that ALCAR improves IFM CPT1 catalysis and thus should be considered as a potential therapy for maintaining cardiac bioenergetics in the aging heart.

Chapter 6

General Conclusions

6.1.1 General Conclusions

While cardiac mitochondrial decay with increasing age is well established, progress in discerning the mechanism(s) involved has been extremely slow. For more than three decades, deterioration of mitochondrial function has been recognized as one of the key subcellular processes that adversely affect myocyte viability, and ultimately cause myocardial hypertrophy and impair ventricular performance in the aging animal. Nevertheless, the root causes of the age-related decline in mitochondrial energy metabolism; specifically, lower fatty acid utilization and ETC dysfunction, which impair myocyte bioenergetics in particular and cardiac reserve in general, are still a matter of controversy. Consequently, there has been no efficient means of limiting the progression of mitochondrial deterioration and hence the loss of cardiac bioenergetics in the aging heart.

In an effort to provide new insights as to the molecular events that adversely affect mitochondrial function and cardiac energy metabolism in the aged myocardium, a side-by-side analysis of age-associated changes in mitochondria from two different subcellular localities was used as the primary experimental approach of this dissertation. Specifically, both deterioration of electron transport supercomplexes and impaired fatty acid-

driven respiration were investigated in two mitochondrial subpopulations in the aging rat heart. The importance of the data presented lies in characterizing those alterations, in light of the intrinsic differences in subcellular locality, as potential causes of the well-known aging phenotypes of cardiac mitochondria: lower bioenergetic reserve capacity, enhanced superoxide production, and oxidative damage [78, 207, 208, 268].

As we discussed in Chapter 1, increasing evidence from structural and functional studies supports the hypothesis that the ETC complexes actually form solid-state macromolecular assemblies where defective supercomplex organization results in pathologies as varied as heart failure [138, 144], Barth syndrome [149], and Leigh syndrome [134, 135]. Moreover, as these pathological conditions are reminiscent of aging, those observations provide a rationale that alterations in supercomplexes are partly responsible for the loss of ETC function characteristic of the aged myocardium. The data presented in Chapters 2 and 4 show that two different subpopulations of rat heart mitochondria contain ETC supercomplexes. Particularly, results from BN-PAGE analysis show that both SSM and IFM display supercomplexes comprised of various stoichiometries of complexes I, III and IV, which typically organize as high mass (1500-2300 kDa) assemblies containing up to four copies of complex

IV. Interestingly, age-related supercomplex deficits were observed in both cardiac mitochondrial subpopulations. In particular, $I_1III_2IV_2$, $I_1III_2IV_3$ and $I_1III_2IV_4$ supercomplexes (1900-2300 kDa, respectively) were found to decline in IFM on an age basis. Thus, because of the complex stoichiometry of these macromolecular assemblies, which suggests more efficient electron transfer to oxygen due to the presence of several copies of complex IV, it is plausible to hypothesize that the age-associated dysfunction of the ETC that specifically affects IFM [23] partially stems from destabilization of supercomplexes of the highest order. Also, these results provide a rationale to further hypothesize that such supercomplex deficits in IFM play an important role in the myocyte loss that affects the aging myocardium [15]. In support of this hypothesis, Oswald et al. showed that impaired cell growth strongly correlates with severe deterioration of supercomplexes comprising more than one copy of complex IV in HeLa cells knocked down for *COX17* [146], which suggests that supercomplexes with the highest masses are critical for maintaining aerobic energy metabolism and cellular function.

In contrast to IFM, the aging defects of SSM supercomplexes were mainly confined to the $I_1III_2IV_1$ supercomplex (1700 kDa), while the higher molecular weight supercomplex assemblies that were most affected in IFM

(i.e. $I_1III_2IV_N$, $N \geq 2$) were not significantly altered. Therefore, results from this dissertation indicate for the first time, that age-related deficits of supercomplexes are asymmetrical with respect to different mitochondrial subcellular locations in the myocyte. Furthermore, because supercomplex deterioration mainly affects IFM, the mitochondrial population that supplies energy for maintaining the contractile function of the myocardium, our results support the hypothesis that age-related supercomplex deficits in IFM constitute one the molecular causes of the loss of cardiac reserve in aging.

A further interpretation of our results is that the apparent asymmetry in supercomplex deterioration may partially explain the evident differences in how aging affects mitochondrial bioenergetics in IFM and SSM, which is not fully understood yet. Specifically, an interpretation of these results in light of the so-called "respiratory string model" proposed by Schägger and colleagues [118-120] may explain previous observations that electron transport characteristics of IFM are more adversely affected with age than SSM [23, 25]. According to this model, the ETC exists as long super-assemblies that wrap the cristae and efficiently transfer electrons to O_2. If this model is correct, then the supercomplexes noted by BN-PAGE are themselves fragments of even higher ordered ETC strings [120, 213]. Therefore, on the basis of this model, it is plausible to hypothesize that the

age-related loss of ETC activity in IFM stems from destabilization of respiratory strings in this mitochondrial subpopulation. On the other hand, oxygen consumption characteristics of SSM may be preserved as a result of maintenance of proper ETC assembly into respiratory strings. In an extension of this interpretation, it is enticing to hypothesize that age-related deterioration of respiratory strings may also be an underlying factor of the higher rates of ROS appearance observed in IFM, relative to SSM [26].

As we described in Chapter 3, several studies indicate that alterations of the IMM proteins (e.g. complex IV) and/or certain phospholipids (e.g. cardiolipin) may be important factors to maintaining supercomplex organization in cardiac mitochondria. Nevertheless, the age-related mechanism(s) of supercomplex deterioration in post-mitotic tissues in general and in cardiac tissue in particular, are not fully known. In Chapters 3 and 4 we show that the lipid organization of the IMM may be an important factor for regulating supercomplex organization. Specifically, in Chapter 4 we present evidence to support the hypothesis that age-related alterations in the content of cardiolipin and its predominant molecular species [i.e. $(18:2)_4$-cardiolipin] may be an important cause of supercomplex deterioration in the rat heart. Nevertheless, results from this dissertation also indicate that supercomplex assembly and organization in

cardiac mitochondria does not solely depend on alterations of cardiolipin and that other IMM constituents need to be investigated with respect to the role they play in promoting age-related supercomplex deficits. Unfortunately, technical limitations due to limited purified supercomplex material prevented a proteomic characterization of potential changes to protein constituents of the IMM (e.g. the Rcf1-type protein and/or protein oxidation) that might be involved in the age-related destabilization of supercomplexes. Further work is necessary to elucidate how such alterations may affect supercomplex organization in the aged heart. In this regard, we have preliminary evidence that suggests that administration of ALCAR to old animals, the micronutrient used in Chapter 5, may promote supercomplex stability in cardiac mitochondria. These observations require future investigation as they may provide new insights as to how age-related alterations of mitochondrial energy metabolism (e.g. fatty acid oxidation) and/or structural organization of the IMM (e.g. phospholipid-protein interactions) affect supercomplex organization and stability. In addition, if these observations are correct, a new role of ALCAR as a micronutrient that improves mitochondrial bioenergetics would complement the therapeutic function of this compound presented in Chapter 5. It is also clear that potential implications of supercomplex destabilization on mitochondrial

function, particularly in the context of cardiac aging, were not explored in this dissertation and deserve future research efforts. Specifically, new approaches should be implemented to investigate functional consequences of supercomplex destabilization *in vitro*. In particular, we have conducted preliminary studies to use rat heart myoblasts (H9c2 cells) as an intact-cell model to explore consequences of supercomplex deficits. Specifically, we have conducted some experiments aimed to explore potential changes in supercomplex organization upon differentiation of H9c2 myoblasts into myotubes. Potential success in using this approach would open the door to use new technologies (e.g. Seahorse analysis of cellular respiration) to explore supercomplex organization in intact cells. A similar experimental scheme could also be implemented using isolated rat cardiomyocytes; however, as cellular yield tend to be relatively low when isolating cells from adult rat hearts, this possibility may be technically more difficult.

Also, it is important to interpret our results for age-related deficits in supercomplexes, cardiolipin and CPT1 activity (see below) in IFM in the context of previous studies from our laboratory where aging was found to correlate with higher levels of ROS appearance in isolated cardiac myocytes in general [58] and in IFM in particular [26]. Taken together, our results suggest that an age-related accumulation of molecular defects specifically

impair IFM bioenergetics, which could adversely affect the energy supply needed to maintain myofibril relaxation and contractile function. In light of the previous findings from our laboratory, such alterations of the molecular components of IFM could stem, at least in part, from increased oxidative damage that specifically affects this mitochondrial subpopulation.

Finally, in Chapter 5 we present the first characterization of age-associated changes in CPT1 activity in the context of two different populations of cardiac mitochondria. In this regard, one of the important findings of this work is that it shows that the aging lesion for fatty acid utilization is the selective alteration of the CPT1 enzyme in IFM and does not appear to be caused by decrements in myocardial L-carnitine. Specifically, our results indicate that the age-related loss of CPT1 activity in IFM is caused by a decrease in the catalytic efficiency of this enzyme to use palmitoyl-CoA as a substrate, with no age-related changes in enzyme kinetics with respect to L-carnitine. It is noteworthy, that specific protein modification(s) that may lead to the aging lesion in IFM CPT1 catalysis were not studied in this dissertation and should be further investigated as they may provide new insights to develop additional therapies that could be used in conjunction with ALCAR to remediate impaired fatty acid metabolism in cardiac aging.

Thus, this dissertation constitutes a study of age-associated alterations in the macromolecular organization of the IMM components that could partly explain the loss of mitochondrial aerobic metabolism in the aging heart. In addition, the characterization of an age-related lesion of the controlling enzyme for cardiac fatty acid oxidation, which selectively affects the mitochondrial subpopulation that appears to be more important for maintenance of the contraction-relaxation cycle, is presented as another important factor that limits mitochondrial function and energy metabolism in the aged myocardium.

Bibliography

[1] Fredriksen PM, Veldtman G, Hechter S, Therrien J, Chen A, Warsi MA, et al. Aerobic capacity in adults with various congenital heart diseases. Am J Cardiol 2001;87:310-4.

[2] Fleg JL, Morrell CH, Bos AG, Brant LJ, Talbot LA, Wright JG, et al. Accelerated longitudinal decline of aerobic capacity in healthy older adults. Circulation 2005;112:674-82.

[3] Kalogeropoulos A, Georgiopoulou V, Kritchevsky SB, Psaty BM, Smith NL, Newman AB, et al. Epidemiology of incident heart failure in a contemporary elderly cohort: the health, aging, and body composition study. Arch Intern Med 2009;169:708-15.

[4] Kannel WB. Incidence and epidemiology of heart failure. Heart Fail Rev 2000;5:167-73.

[5] Masoudi FA, Havranek EP, Krumholz HM. The burden of chronic congestive heart failure in older persons: magnitude and implications for policy and research. Heart Fail Rev 2002;7:9-16.

[6] Nagamine M, Jiang HJ, Merrill CT. Trends in elderly hospitalizations, 1997-2004. Statistical Brief Agency for Healthcare Research and Quality, Rockville, MD 2006;14.

[7] Russo CA, Elixhauser A. Hospitalizations in the elderly population, 2003. Statistical Brief Agency for Healthcare Research and Quality, Rockville, MD 2006;6.

[8] Stranges E KN, Elixhauser A. Components of growth in inpatient hospital costs, 1997-2009. HCUP Statistical Brief Agency for Healthcare Research and Quality, Rockville, MD 2011;123.

[9] Hacker TA, McKiernan SH, Douglas PS, Wanagat J, Aiken JM. Age-related changes in cardiac structure and function in Fischer 344 x Brown Norway hybrid rats. Am J Physiol Heart Circ Physiol 2006;290:H304-11.

[10] Lieber SC, Aubry N, Pain J, Diaz G, Kim SJ, Vatner SF. Aging increases stiffness of cardiac myocytes measured by atomic force microscopy nanoindentation. Am J Physiol Heart Circ Physiol 2004;287:H645-51.

[11] Boyle AJ, Shih H, Hwang J, Ye J, Lee B, Zhang Y, et al. Cardiomyopathy of aging in the mammalian heart is characterized by myocardial hypertrophy, fibrosis and a predisposition towards

cardiomyocyte apoptosis and autophagy. Exp Gerontol 2011;46:549-59.

[12] Schocke MF, Metzler B, Wolf C, Steinboeck P, Kremser C, Pachinger O, et al. Impact of aging on cardiac high-energy phosphate metabolism determined by phosphorus-31 2-dimensional chemical shift imaging (31P 2D CSI). Magn Reson Imaging 2003;21:553-9.

[13] Cheng S, Fernandes VR, Bluemke DA, McClelland RL, Kronmal RA, Lima JA. Age-related left ventricular remodeling and associated risk for cardiovascular outcomes: the Multi-Ethnic Study of Atherosclerosis. Circ Cardiovasc Imaging 2009;2:191-8.

[14] Gates PE, Tanaka H, Graves J, Seals DR. Left ventricular structure and diastolic function with human ageing. Relation to habitual exercise and arterial stiffness. Eur Heart J 2003;24:2213-20.

[15] Kajstura J, Gurusamy N, Ogorek B, Goichberg P, Clavo-Rondon C, Hosoda T, et al. Myocyte turnover in the aging human heart. Circ Res 2010;107:1374-86.

[16] Page E, McCallister LP. Quantitative electron microscopic description of heart muscle cells. Application to normal, hypertrophied and thyroxin-stimulated hearts. Am J Cardiol 1973;31:172-81.

[17] Kahles H, Hellige G, Hunneman DH, Mezger VA, Bretschneider HJ. Influence of myocardial substrate utilization on the oxygen consumption of the heart. Clin Cardiol 1982;5:286-93.

[18] Saddik M, Lopaschuk GD. Myocardial triglyceride turnover and contribution to energy substrate utilization in isolated working rat hearts. J Biol Chem 1991;266:8162-70.

[19] Lukyanenko V, Chikando A, Lederer WJ. Mitochondria in cardiomyocyte Ca2+ signaling. Int J Biochem Cell Biol 2009;41:1957-71.

[20] Palmer JW, Tandler B, Hoppel CL. Heterogeneous response of subsarcolemmal heart mitochondria to calcium. Am J Physiol 1986;250:H741-8.

[21] Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res 2004;61:372-85.

[22] Bodyak N, Kang PM, Hiromura M, Sulijoadikusumo I, Horikoshi N, Khrapko K, et al. Gene expression profiling of the aging mouse cardiac myocytes. Nucleic Acids Res 2002;30:3788-94.

[23] Fannin SW, Lesnefsky EJ, Slabe TJ, Hassan MO, Hoppel CL. Aging selectively decreases oxidative capacity in rat heart interfibrillar mitochondria. Arch Biochem Biophys 1999;372:399-407.

[24] Lenaz G, Bovina C, Castelluccio C, Fato R, Formiggini G, Genova ML, et al. Mitochondrial complex I defects in aging. Mol Cell Biochem 1997;174:329-33.

[25] Lesnefsky EJ, Gudz TI, Moghaddas S, Migita CT, Ikeda-Saito M, Turkaly PJ, et al. Aging decreases electron transport complex III activity in heart interfibrillar mitochondria by alteration of the cytochrome c binding site. J Mol Cell Cardiol 2001;33:37-47.

[26] Suh JH, Heath SH, Hagen TM. Two subpopulations of mitochondria in the aging rat heart display heterogenous levels of oxidative stress. Free Radic Biol Med 2003;35:1064-72.

[27] Bratic I, Trifunovic A. Mitochondrial energy metabolism and ageing. Biochim Biophys Acta 2010;1797:961-7.

[28] Hagen TM, Yowe DL, Bartholomew JC, Wehr CM, Do KL, Park JY, et al. Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. Proc Natl Acad Sci USA 1997;94:3064-9.

[29] Hayakawa M, Sugiyama S, Hattori K, Takasawa M, Ozawa T. Age-associated damage in mitochondrial DNA in human hearts. Mol Cell Biochem 1993;119:95-103.

[30] Castro Mdel R, Suarez E, Kraiselburd E, Isidro A, Paz J, Ferder L, et al. Aging increases mitochondrial DNA damage and oxidative stress in liver of rhesus monkeys. Exp Gerontol 2012;47:29-37.

[31] Pak JW, Herbst A, Bua E, Gokey N, McKenzie D, Aiken JM. Mitochondrial DNA mutations as a fundamental mechanism in physiological declines associated with aging. Aging cell 2003;2:1-7.

[32] Cooper JM, Mann VM, Schapira AH. Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. J Neurol Sci 1992;113:91-8.

[33] Lai LP, Tsai CC, Su MJ, Lin JL, Chen YS, Tseng YZ, et al. Atrial fibrillation is associated with accumulation of aging-related common type mitochondrial DNA deletion mutation in human atrial tissue. Chest 2003;123:539-44.

[34] Brunk UT, Terman A. The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. Eur J Biochem 2002;269:1996-2002.

[35] Muster B, Kohl W, Wittig I, Strecker V, Joos F, Haase W, et al. Respiratory chain complexes in dynamic mitochondria display a patchy distribution in life cells. PLoS One 2010;5:e11910.
[36] Weber TA, Reichert AS. Impaired quality control of mitochondria: aging from a new perspective. Exp Gerontol 2010;45:503-11.
[37] Terman A, Dalen H, Eaton JW, Neuzil J, Brunk UT. Mitochondrial recycling and aging of cardiac myocytes: the role of autophagocytosis. Exp Gerontol 2003;38:863-76.
[38] Terman A, Brunk UT. Myocyte aging and mitochondrial turnover. Exp Gerontol 2004;39:701-5.
[39] Cui H, Kong Y, Zhang H. Oxidative stress, mitochondrial dysfunction, and aging. J Signal Transduct 2012;2012:646354.
[40] Marchi S, Giorgi C, Suski JM, Agnoletto C, Bononi A, Bonora M, et al. Mitochondria-ros crosstalk in the control of cell death and aging. J Signal Transduct 2012;2012:329635.
[41] Greco M, Villani G, Mazzucchelli F, Bresolin N, Papa S, Attardi G. Marked aging-related decline in efficiency of oxidative phosphorylation in human skin fibroblasts. FASEB J 2003;17:1706-8.
[42] Marcinek DJ, Schenkman KA, Ciesielski WA, Lee D, Conley KE. Reduced mitochondrial coupling in vivo alters cellular energetics in aged mouse skeletal muscle. J Physiol 2005;569:467-73.
[43] Kostler H, Landschutz W, Koeppe S, Seyfarth T, Lipke C, Sandstede J, et al. Age and gender dependence of human cardiac phosphorus metabolites determined by SLOOP 31P MR spectroscopy. Magn Reson Med 2006;56:907-11.
[44] Kates AM, Herrero P, Dence C, Soto P, Srinivasan M, Delano DG, et al. Impact of aging on substrate metabolism by the human heart. J Am Coll Cardiol 2003;41:293-9.
[45] Boveris A, Navarro A. Brain mitochondrial dysfunction in aging. IUBMB Life 2008;60:308-14.
[46] Navarro A, Lopez-Cepero JM, Bandez MJ, Sanchez-Pino MJ, Gomez C, Cadenas E, et al. Hippocampal mitochondrial dysfunction in rat aging. Am J Physiol Regul Integr Comp Physiol 2008;294:R501-9.
[47] Chen JC, Warshaw JB, Sanadi DR. Regulation of mitochondrial respiration in senescence. J Cell Physiol 1972;80:141-8.
[48] Kim JH, Woldgiorgis G, Elson CE, Shrago E. Age-related changes in respiration coupled to phosphorylation. I. Hepatic mitochondria. Mech Ageing Dev 1988;46:263-77.

[49] O'Toole JF, Patel HV, Naples CJ, Fujioka H, Hoppel CL. Decreased cytochrome c mediates an age-related decline of oxidative phosphorylation in rat kidney mitochondria. Biochem J 2010;427:105-12.

[50] Jahangir A, Ozcan C, Holmuhamedov EL, Terzic A. Increased calcium vulnerability of senescent cardiac mitochondria: protective role for a mitochondrial potassium channel opener. Mech Ageing Dev 2001;122:1073-86.

[51] Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, et al. Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci U S A 2005;102:5618-23.

[52] Paradies G, Ruggiero FM. Age-related changes in the activity of the pyruvate carrier and in the lipid composition in rat-heart mitochondria. Biochim Biophys Acta 1990;1016:207-12.

[53] Mather M, Rottenberg H. Aging enhances the activation of the permeability transition pore in mitochondria. Biochem Biophys Res Commun 2000;273:603-8.

[54] Hofer T, Servais S, Seo AY, Marzetti E, Hiona A, Upadhyay SJ, et al. Bioenergetics and permeability transition pore opening in heart subsarcolemmal and interfibrillar mitochondria: effects of aging and lifelong calorie restriction. Mech Ageing Dev 2009;130:297-307.

[55] Niemann B, Chen Y, Teschner M, Li L, Silber RE, Rohrbach S. Obesity induces signs of premature cardiac aging in younger patients: the role of mitochondria. J Am Coll Cardiol 2011;57:577-85.

[56] Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. Apoptosis 2007;12:913-22.

[57] Brand MD. The sites and topology of mitochondrial superoxide production. Exp Gerontol 2010;45:466-72.

[58] Suh JH, Shigeno ET, Morrow JD, Cox B, Rocha AE, Frei B, et al. Oxidative stress in the aging rat heart is reversed by dietary supplementation with (R)-(alpha)-lipoic acid. FASEB J 2001;15:700-6.

[59] Lesnefsky EJ, Hoppel CL. Cardiolipin as an oxidative target in cardiac mitochondria in the aged rat. Biochim Biophys Acta 2008;1777:1020-7.

[60] Lesnefsky EJ, Minkler P, Hoppel CL. Enhanced modification of cardiolipin during ischemia in the aged heart. J Mol Cell Cardiol 2009;46:1008-15.

[61] Miro O, Casademont J, Casals E, Perea M, Urbano-Marquez A, Rustin P, et al. Aging is associated with increased lipid peroxidation in human hearts, but not with mitochondrial respiratory chain enzyme defects. Cardiovasc Res 2000;47:624-31.
[62] Judge S, Leeuwenburgh C. Cardiac mitochondrial bioenergetics, oxidative stress, and aging. Am J Physiol Cell Physiol 2007;292:C1983-92.
[63] Choksi KB, Boylston WH, Rabek JP, Widger WR, Papaconstantinou J. Oxidatively damaged proteins of heart mitochondrial electron transport complexes. Biochim Biophys Acta 2004;1688:95-101.
[64] Moreau R, Heath SH, Doneanu CE, Lindsay JG, Hagen TM. Age-related increase in 4-hydroxynonenal adduction to rat heart alpha-ketoglutarate dehydrogenase does not cause loss of its catalytic activity. Antioxid Redox Signal 2003;5:517-27.
[65] Paradies G, Petrosillo G, Paradies V, Ruggiero FM. Oxidative stress, mitochondrial bioenergetics, and cardiolipin in aging. Free Radic Biol Med 2010;48:1286-95.
[66] Houtkooper RH, Vaz FM. Cardiolipin, the heart of mitochondrial metabolism. Cell Mol Life Sci 2008;65:2493-506.
[67] Schlame M, Rua D, Greenberg ML. The biosynthesis and functional role of cardiolipin. Prog Lipid Res 2000;39:257-88.
[68] Schlame M. Cardiolipin synthesis for the assembly of bacterial and mitochondrial membranes. J Lipid Res 2008;49:1607-20.
[69] Schlame M, Ren M, Xu Y, Greenberg ML, Haller I. Molecular symmetry in mitochondrial cardiolipins. Chem Phys Lipids 2005;138:38-49.
[70] Tatarkova Z, Kuka S, Racay P, Lehotsky J, Dobrota D, Mistuna D, et al. Effects of aging on activities of mitochondrial electron transport chain complexes and oxidative damage in rat heart. Physiol Res 2011;60:281-9.
[71] Li SY, Du M, Dolence EK, Fang CX, Mayer GE, Ceylan-Isik AF, et al. Aging induces cardiac diastolic dysfunction, oxidative stress, accumulation of advanced glycation endproducts and protein modification. Aging cell 2005;4:57-64.
[72] Kanski J, Behring A, Pelling J, Schoneich C. Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: effects of biological aging. Am J Physiol Heart Circ Physiol 2005;288:H371-81.

[73] Musicco C, Capelli V, Pesce V, Timperio AM, Calvani M, Mosconi L, et al. Accumulation of overoxidized Peroxiredoxin III in aged rat liver mitochondria. Biochim Biophys Acta 2009;1787:890-6.
[74] Bakala H, Delaval E, Hamelin M, Bismuth J, Borot-Laloi C, Corman B, et al. Changes in rat liver mitochondria with aging. Lon protease-like reactivity and N(epsilon)-carboxymethyllysine accumulation in the matrix. Eur J Biochem 2003;270:2295-302.
[75] Sandhu SK, Kaur G. Mitochondrial electron transport chain complexes in aging rat brain and lymphocytes. Biogerontology 2003;4:19-29.
[76] Lenaz G, D'Aurelio M, Merlo Pich M, Genova ML, Ventura B, Bovina C, et al. Mitochondrial bioenergetics in aging. Biochim Biophys Acta 2000;1459:397-404.
[77] Petrosillo G, Matera M, Casanova G, Ruggiero FM, Paradies G. Mitochondrial dysfunction in rat brain with aging Involvement of complex I, reactive oxygen species and cardiolipin. Neurochemistry international 2008;53:126-31.
[78] Choksi KB, Papaconstantinou J. Age-related alterations in oxidatively damaged proteins of mouse heart mitochondrial electron transport chain complexes. Free Radic Biol Med 2008;44:1795-805.
[79] Petrosillo G, Matera M, Moro N, Ruggiero FM, Paradies G. Mitochondrial complex I dysfunction in rat heart with aging: critical role of reactive oxygen species and cardiolipin. Free Radic Biol Med 2009;46:88-94.
[80] Moghaddas S, Hoppel CL, Lesnefsky EJ. Aging defect at the QO site of complex III augments oxyradical production in rat heart interfibrillar mitochondria. Arch Biochem Biophys 2003;414:59-66.
[81] Muller-Hocker J, Schafer S, Link TA, Possekel S, Hammer C. Defects of the respiratory chain in various tissues of old monkeys: a cytochemical-immunocytochemical study. Mech Ageing Dev 1996;86:197-213.
[82] Muller-Hocker J. Cytochrome-c-oxidase deficient cardiomyocytes in the human heart--an age-related phenomenon. A histochemical ultracytochemical study. Am J Pathol 1989;134:1167-73.
[83] Ojaimi J, Masters CL, Opeskin K, McKelvie P, Byrne E. Mitochondrial respiratory chain activity in the human brain as a function of age. Mech Ageing Dev 1999;111:39-47.
[84] Lopez ME, Van Zeeland NL, Dahl DB, Weindruch R, Aiken JM. Cellular phenotypes of age-associated skeletal muscle mitochondrial abnormalities in rhesus monkeys. Mutat Res 2000;452:123-38.

[85] Hansford RG, Castro F. Age-linked changes in the activity of enzymes of the tricarboxylate cycle and lipid oxidation, and of carnitine content, in muscles of the rat. Mech Ageing Dev 1982;19:191-200.

[86] Paradies G, Ruggiero FM, Petrosillo G, Gadaleta MN, Quagliariello E. Effect of aging and acetyl-L-carnitine on the activity of cytochrome oxidase and adenine nucleotide translocase in rat heart mitochondria. FEBS Lett 1994;350:213-5.

[87] Monette JS, Gomez LA, Moreau RF, Dunn KC, Butler JA, Finlay LA, et al. (R)-alpha-Lipoic acid treatment restores ceramide balance in aging rat cardiac mitochondria. Pharmacol Res 2011;63:23-9.

[88] Nohl H, Hegner D. Do mitochondria produce oxygen radicals in vivo? Eur J Biochem 1978;82:563-7.

[89] Barja G. Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. J Bioenerg Biomembr 1999;31:347-66.

[90] Wanagat J, Wolff MR, Aiken JM. Age-associated changes in function, structure and mitochondrial genetic and enzymatic abnormalities in the Fischer 344 x Brown Norway F(1) hybrid rat heart. J Mol Cell Cardiol 2002;34:17-28.

[91] Ohnishi ST, Ohnishi T, Muranaka S, Fujita H, Kimura H, Uemura K, et al. A possible site of superoxide generation in the complex I segment of rat heart mitochondria. J Bioenerg Biomembr 2005;37:1-15.

[92] Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009;417:1-13.

[93] Suh JH, Wang H, Liu RM, Liu J, Hagen TM. (R)-alpha-lipoic acid reverses the age-related loss in GSH redox status in post-mitotic tissues: evidence for increased cysteine requirement for GSH synthesis. Arch Biochem Biophys 2004;423:126-35.

[94] Hagen TM, Ingersoll RT, Wehr CM, Lykkesfeldt J, Vinarsky V, Bartholomew JC, et al. Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. Proc Natl Acad Sci USA 1998;95:9562-6.

[95] Miles MV, Horn PS, Tang PH, Morrison JA, Miles L, DeGrauw T, et al. Age-related changes in plasma coenzyme Q10 concentrations and redox state in apparently healthy children and adults. Clin Chim Acta 2004;347:139-44.

[96] Rosenfeldt FL, Pepe S, Linnane A, Nagley P, Rowland M, Ou R, et al. Coenzyme Q10 protects the aging heart against stress: studies in

rats, human tissues, and patients. Ann N Y Acad Sci 2002;959:355-9; discussion 463-5.

[97] Kalen A, Appelkvist EL, Dallner G. Age-related changes in the lipid compositions of rat and human tissues. Lipids 1989;24:579-84.

[98] Beyer RE, Burnett BA, Cartwright KJ, Edington DW, Falzon MJ, Kreitman KR, et al. Tissue coenzyme Q (ubiquinone) and protein concentrations over the life span of the laboratory rat. Mech Ageing Dev 1985;32:267-81.

[99] Kamzalov S, Sohal RS. Effect of age and caloric restriction on coenzyme Q and alpha-tocopherol levels in the rat. Exp Gerontol 2004;39:1199-205.

[100] Sohal RS, Forster MJ. Coenzyme Q, oxidative stress and aging. Mitochondrion 2007;7 Suppl:S103-11.

[101] Vorbeck ML, Martin AP, Long JW, Jr., Smith JM, Orr RR, Jr. Aging-dependent modification of lipid composition and lipid structural order parameter of hepatic mitochondria. Arch Biochem Biophys 1982;217:351-61.

[102] Paradies G, Petrosillo G, Gadaleta MN, Ruggiero FM. The effect of aging and acetyl-L-carnitine on the pyruvate transport and oxidation in rat heart mitochondria. FEBS Lett 1999;454:207-9.

[103] Paradies G, Ruggiero FM, Petrosillo G, Gadaleta MN, Quagliariello E. Carnitine-acylcarnitine translocase activity in cardiac mitochondria from aged rats: the effect of acetyl-L-carnitine. Mech Ageing Dev 1995;84:103-12.

[104] Nohl H, Kramer R. Molecular basis of age-dependent changes in the activity of adenine nucleotide translocase. Mech Ageing Dev 1980;14:137-44.

[105] Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Age-dependent decline in the cytochrome c oxidase activity in rat heart mitochondria: role of cardiolipin. FEBS Lett 1997;406:136-8.

[106] Lewin MB, Timiras PS. Lipid changes with aging in cardiac mitochondrial membranes. Mech Ageing Dev 1984;24:343-51.

[107] McMillin JB, Taffet GE, Taegtmeyer H, Hudson EK, Tate CA. Mitochondrial metabolism and substrate competition in the aging Fischer rat heart. Cardiovasc Res 1993;27:2222-8.

[108] Hagen TM, Wehr CM, Ames BN. Mitochondrial decay in aging. Reversal through supplementation of acetyl-L-carnitine and N-tert-butyl-alpha-phenyl-nitrone. Ann NY Acad Sci 1998;854:214-23.

[109] Moghaddas S, Stoll MS, Minkler PE, Salomon RG, Hoppel CL, Lesnefsky EJ. Preservation of cardiolipin content during aging in rat

heart interfibrillar mitochondria. J Gerontol A Biol Sci Med Sci 2002;57:B22-8.

[110] Helmy FM, Hack MH, Juracka A. Age-related changes of the endogenous cardiolipin and plasmalogens of guinea pig kidney and their in vitro hydrolysis by endogenous phospholipases: a thin layer chromatographic analysis in conjunction with densitometric measurement. Cell Biochem Funct 2003;21:337-44.

[111] Malhotra A, Xu Y, Ren M, Schlame M. Formation of molecular species of mitochondrial cardiolipin. 1. A novel transacylation mechanism to shuttle fatty acids between sn-1 and sn-2 positions of multiple phospholipid species. Biochim Biophys Acta 2009;1791:314-20.

[112] Schlame M, Shanske S, Doty S, Konig T, Sculco T, DiMauro S, et al. Microanalysis of cardiolipin in small biopsies including skeletal muscle from patients with mitochondrial disease. J Lipid Res 1999;40:1585-92.

[113] Lee HJ, Mayette J, Rapoport SI, Bazinet RP. Selective remodeling of cardiolipin fatty acids in the aged rat heart. Lipids in health and disease 2006;5:2.

[114] Hackenbrock CR, Chazotte B, Gupte SS. The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport. J Bioenerg Biomembr 1986;18:331-68.

[115] Chazotte B, Hackenbrock CR. The multicollisional, obstructed, long-range diffusional nature of mitochondrial electron transport. J Biol Chem 1988;263:14359-67.

[116] Gupte SS, Hackenbrock CR. The role of cytochrome c diffusion in mitochondrial electron transport. J Biol Chem 1988;263:5248-53.

[117] Gupte SS, Hackenbrock CR. Multidimensional diffusion modes and collision frequencies of cytochrome c with its redox partners. J Biol Chem 1988;263:5241-7.

[118] Schagger H, Pfeiffer K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. EMBO J 2000;19:1777-83.

[119] Wittig I, Carrozzo R, Santorelli FM, Schagger H. Supercomplexes and subcomplexes of mitochondrial oxidative phosphorylation. Biochim Biophys Acta 2006;1757:1066-72.

[120] Wittig I, Schagger H. Supramolecular organization of ATP synthase and respiratory chain in mitochondrial membranes. Biochim Biophys Acta 2009;1787:672-80.

[121] Acin-Perez R, Fernandez-Silva P, Peleato ML, Perez-Martos A, Enriquez JA. Respiratory active mitochondrial supercomplexes. Molecular cell 2008;32:529-39.
[122] Bianchi C, Genova ML, Parenti Castelli G, Lenaz G. The mitochondrial respiratory chain is partially organized in a supercomplex assembly: kinetic evidence using flux control analysis. J Biol Chem 2004;279:36562-9.
[123] Genova ML, Baracca A, Biondi A, Casalena G, Faccioli M, Falasca AI, et al. Is supercomplex organization of the respiratory chain required for optimal electron transfer activity? Biochim Biophys Acta 2008;1777:740-6.
[124] Lenaz G, Baracca A, Barbero G, Bergamini C, Dalmonte ME, Del Sole M, et al. Mitochondrial respiratory chain super-complex I-III in physiology and pathology. Biochim Biophys Acta 2010;1797:633-40.
[125] Lenaz G, Genova ML. Kinetics of integrated electron transfer in the mitochondrial respiratory chain: random collisions vs. solid state electron channeling. Am J Physiol Cell Physiol 2007;292:C1221-39.
[126] Lenaz G, Genova ML. Structural and functional organization of the mitochondrial respiratory chain: a dynamic super-assembly. Int J Biochem Cell Biol 2009;41:1750-72.
[127] Lenaz G, Genova ML. Mobility and function of coenzyme Q (ubiquinone) in the mitochondrial respiratory chain. Biochim Biophys Acta 2009;1787:563-73.
[128] Althoff T, Mills DJ, Popot JL, Kuhlbrandt W. Arrangement of electron transport chain components in bovine mitochondrial supercomplex I1III2IV1. EMBO J 2011;30:4652-64.
[129] Dudkina NV, Kudryashev M, Stahlberg H, Boekema EJ. Interaction of complexes I, III, and IV within the bovine respirasome by single particle cryoelectron tomography. Proc Natl Acad Sci U S A 2011;108:15196-200.
[130] Reifschneider NH, Goto S, Nakamoto H, Takahashi R, Sugawa M, Dencher NA, et al. Defining the mitochondrial proteomes from five rat organs in a physiologically significant context using 2D blue-native/SDS-PAGE. Journal of proteome research 2006;5:1117-32.
[131] Wittig I, Schagger H. Advantages and limitations of clear-native PAGE. Proteomics 2005;5:4338-46.
[132] Gomez LA, Monette JS, Chavez JD, Maier CS, Hagen TM. Supercomplexes of the mitochondrial electron transport chain

decline in the aging rat heart. Arch Biochem Biophys 2009;490:30-5.

[133] Moreno-Lastres D, Fontanesi F, Garcia-Consuegra I, Martin MA, Arenas J, Barrientos A, et al. Mitochondrial complex I plays an essential role in human respirasome assembly. Cell Metab 2012;15:324-35.

[134] Schagger H, de Coo R, Bauer MF, Hofmann S, Godinot C, Brandt U. Significance of respirasomes for the assembly/stability of human respiratory chain complex I. J Biol Chem 2004;279:36349-53.

[135] McKenzie M, Lazarou M, Thorburn DR, Ryan MT. Analysis of mitochondrial subunit assembly into respiratory chain complexes using Blue Native polyacrylamide gel electrophoresis. Anal Biochem 2007;364:128-37.

[136] D'Aurelio M, Gajewski CD, Lenaz G, Manfredi G. Respiratory chain supercomplexes set the threshold for respiration defects in human mtDNA mutant cybrids. Human molecular genetics 2006;15:2157-69.

[137] Minkler PE, Hoppel CL. Separation and characterization of cardiolipin molecular species by reverse-phase ion pair high-performance liquid chromatography-mass spectrometry. J Lipid Res 2010;51:856-65.

[138] Rosca M, Minkler P, Hoppel CL. Cardiac mitochondria in heart failure: normal cardiolipin profile and increased threonine phosphorylation of complex IV. Biochim Biophys Acta 2011;1807:1373-82.

[139] Ritov VB, Menshikova EV, Kelley DE. Analysis of cardiolipin in human muscle biopsy. J Chromatogr B Analyt Technol Biomed Life Sci 2006;831:63-71.

[140] Valianpour F, Mitsakos V, Schlemmer D, Towbin JA, Taylor JM, Ekert PG, et al. Monolysocardiolipins accumulate in Barth syndrome but do not lead to enhanced apoptosis. J Lipid Res 2005;46:1182-95.

[141] Valianpour F, Wanders RJ, Overmars H, Vreken P, Van Gennip AH, Baas F, et al. Cardiolipin deficiency in X-linked cardioskeletal myopathy and neutropenia (Barth syndrome, MIM 302060): a study in cultured skin fibroblasts. J Pediatr 2002;141:729-33.

[142] Vempati UD, Han X, Moraes CT. Lack of cytochrome c in mouse fibroblasts disrupts assembly/stability of respiratory complexes I and IV. J Biol Chem 2009;284:4383-91.

[143] Gomez LA, Monette JS, Chavez JD, Maier CS, Hagen TM. Supercomplexes of the mitochondrial electron transport chain decline in the aging rat heart. Arch Biochem Biophys 2009.
[144] Rosca MG, Vazquez EJ, Kerner J, Parland W, Chandler MP, Stanley W, et al. Cardiac mitochondria in heart failure: decrease in respirasomes and oxidative phosphorylation. Cardiovasc Res 2008;80:30-9.
[145] Chen YC, Taylor EB, Dephoure N, Heo JM, Tonhato A, Papandreou I, et al. Identification of a protein mediating respiratory supercomplex stability. Cell Metab 2012;15:348-60.
[146] Oswald C, Krause-Buchholz U, Rodel G. Knockdown of human COX17 affects assembly and supramolecular organization of cytochrome c oxidase. J Mol Biol 2009;389:470-9.
[147] Fornuskova D, Stiburek L, Wenchich L, Vinsova K, Hansikova H, Zeman J. Novel insights into the assembly and function of human nuclear-encoded cytochrome c oxidase subunits 4, 5a, 6a, 7a and 7b. Biochem J 2010;428:363-74.
[148] van Raam BJ, Sluiter W, de Wit E, Roos D, Verhoeven AJ, Kuijpers TW. Mitochondrial membrane potential in human neutrophils is maintained by complex III activity in the absence of supercomplex organisation. PLoS One 2008;3:e2013.
[149] McKenzie M, Lazarou M, Thorburn DR, Ryan MT. Mitochondrial respiratory chain supercomplexes are destabilized in Barth Syndrome patients. J Mol Biol 2006;361:462-9.
[150] Diaz F, Fukui H, Garcia S, Moraes CT. Cytochrome c oxidase is required for the assembly/stability of respiratory complex I in mouse fibroblasts. Mol Cell Biol 2006;26:4872-81.
[151] Lazarou M, Smith SM, Thorburn DR, Ryan MT, McKenzie M. Assembly of nuclear DNA-encoded subunits into mitochondrial complex IV, and their preferential integration into supercomplex forms in patient mitochondria. FEBS J 2009;276:6701-13.
[152] Schlame M, Towbin JA, Heerdt PM, Jehle R, DiMauro S, Blanck TJ. Deficiency of tetralinoleoyl-cardiolipin in Barth syndrome. Ann Neurol 2002;51:634-7.
[153] Schlame M, Brody S, Hostetler KY. Mitochondrial cardiolipin in diverse eukaryotes. Comparison of biosynthetic reactions and molecular acyl species. Eur J Biochem 1993;212:727-35.
[154] Frenzel M, Rommelspacher H, Sugawa MD, Dencher NA. Ageing alters the supramolecular architecture of OxPhos complexes in rat brain cortex. Exp Gerontol 2010;45:563-72.

[155] De los Rios Castillo D, Zarco-Zavala M, Olvera-Sanchez S, Pardo JP, Juarez O, Martinez F, et al. Atypical cristae morphology of human syncytiotrophoblast mitochondria: role for complex V. J Biol Chem 2011;286:23911-9.
[156] Marques I, Dencher NA, Videira A, Krause F. Supramolecular organization of the respiratory chain in Neurospora crassa mitochondria. Eukaryotic cell 2007;6:2391-405.
[157] Suthammarak W, Yang YY, Morgan PG, Sedensky MM. Complex I function is defective in complex IV-deficient Caenorhabditis elegans. J Biol Chem 2009;284:6425-35.
[158] Krause F, Scheckhuber CQ, Werner A, Rexroth S, Reifschneider NH, Dencher NA, et al. OXPHOS Supercomplexes: respiration and life-span control in the aging model Podospora anserina. Ann N Y Acad Sci 2006;1067:106-15.
[159] Schagger H. Respiratory chain supercomplexes of mitochondria and bacteria. Biochim Biophys Acta 2002;1555:154-9.
[160] Bultema JB, Braun HP, Boekema EJ, Kouril R. Megacomplex organization of the oxidative phosphorylation system by structural analysis of respiratory supercomplexes from potato. Biochim Biophys Acta 2009;1787:60-7.
[161] Krause F, Reifschneider NH, Vocke D, Seelert H, Rexroth S, Dencher NA. "Respirasome"-like supercomplexes in green leaf mitochondria of spinach. J Biol Chem 2004;279:48369-75.
[162] Sunderhaus S, Klodmann J, Lenz C, Braun HP. Supramolecular structure of the OXPHOS system in highly thermogenic tissue of Arum maculatum. Plant Physiol Biochem 2010;48:265-72.
[163] Eubel H, Jansch L, Braun HP. New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of complex II. Plant Physiol 2003;133:274-86.
[164] Chien LF, Wu YC, Chen HP. Mitochondrial energy metabolism in young bamboo rhizomes from Bambusa oldhamii and Phyllostachys edulis during shooting stage. Plant Physiol Biochem 2011;49:449-57.
[165] Peters K, Dudkina NV, Jansch L, Braun HP, Boekema EJ. A structural investigation of complex I and I+III2 supercomplex from Zea mays at 11-13 A resolution: assignment of the carbonic anhydrase domain and evidence for structural heterogeneity within complex I. Biochim Biophys Acta 2008;1777:84-93.

[166] Kahles H, Hellige G, Hunneman DH, Mezger VA, Bretschneider HJ. Influence of myocardial substrate utilization on the oxygen consumption of the heart. Clin Cardiol 1982;5:286-93.
[167] Hansford RG. Lipid oxidation by heart mitochondria from young adult and senescent rats. Biochem J 1978;170:285-95.
[168] Abu-Erreish GM, Neely JR, Whitmer JT, Whitman V, Sanadi DR. Fatty acid oxidation by isolated perfused working hearts of aged rats. Am J Physiol 1977;232:E258-62.
[169] Hyyti OM, Ledee D, Ning XH, Ge M, Portman MA. Aging impairs myocardial fatty acid and ketone oxidation and modifies cardiac functional and metabolic responses to insulin in mice. Am J Physiol Heart Circ Physiol 2010;299:H868-75.
[170] van der Meer RW, Rijzewijk LJ, Diamant M, Hammer S, Schar M, Bax JJ, et al. The ageing male heart: myocardial triglyceride content as independent predictor of diastolic function. Eur Heart J 2008;29:1516-22.
[171] Lewin TM, Coleman RA. Regulation of myocardial triacylglycerol synthesis and metabolism. Biochim Biophys Acta 2003;1634:63-75.
[172] Sharma S, Adrogue JV, Golfman L, Uray I, Lemm J, Youker K, et al. Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. FASEB J 2004;18:1692-700.
[173] Koonen DP, Febbraio M, Bonnet S, Nagendran J, Young ME, Michelakis ED, et al. CD36 expression contributes to age-induced cardiomyopathy in mice. Circulation 2007;116:2139-47.
[174] Slawik M, Vidal-Puig AJ. Lipotoxicity, overnutrition and energy metabolism in aging. Ageing Res Rev 2006;5:144-64.
[175] Wende AR, Abel ED. Lipotoxicity in the heart. Biochim Biophys Acta 2010;1801:311-9.
[176] Brindley DN, Kok BP, Kienesberger PC, Lehner R, Dyck JR. Shedding light on the enigma of myocardial lipotoxicity: the involvement of known and putative regulators of fatty acid storage and mobilization. Am J Physiol Endocrinol Metab 2010;298:E897-908.
[177] Odiet JA, Boerrigter ME, Wei JY. Carnitine palmitoyl transferase-I activity in the aging mouse heart. Mech Ageing Dev 1995;79:127-36.
[178] Lee CK, Allison DB, Brand J, Weindruch R, Prolla TA. Transcriptional profiles associated with aging and middle age-onset caloric restriction in mouse hearts. Proc Natl Acad Sci USA 2002;99:14988-93.

[179] Ramsay RR, Gandour RD, van der Leij FR. Molecular enzymology of carnitine transfer and transport. Biochim Biophys Acta 2001;1546:21-43.
[180] McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 1997;244:1-14.
[181] Bartlett K, Eaton S. Mitochondrial beta-oxidation. Eur J Biochem 2004;271:462-9.
[182] Kim JY, Kim DH, Choi J, Park JK, Jeong KS, Leeuwenburgh C, et al. Changes in lipid distribution during aging and its modulation by calorie restriction. Age 2009;31:127-42.
[183] Dobbins RL, Szczepaniak LS, Bentley B, Esser V, Myhill J, McGarry JD. Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats. Diabetes 2001;50:123-30.
[184] Rebouche CJ, Seim H. Carnitine metabolism and its regulation in microorganisms and mammals. Annu Rev Nutr 1998;18:39-61.
[185] Costell M, O'Connor JE, Grisolia S. Age-dependent decrease of carnitine content in muscle of mice and humans. Biochem Biophys Res Commun 1989;161:1135-43.
[186] Opalka JR, Gellerich FN, Zierz S. Age and sex dependency of carnitine concentration in human serum and skeletal muscle. Clin Chem 2001;47:2150-3.
[187] Maccari F, Arseni A, Chiodi P, Ramacci MT, Angelucci L. Levels of carnitines in brain and other tissues of rats of different ages: effect of acetyl-L-carnitine administration. Exp Gerontol 1990;25:127-34.
[188] Tanaka Y, Sasaki R, Fukui F, Waki H, Kawabata T, Okazaki M, et al. Acetyl-L-carnitine supplementation restores decreased tissue carnitine levels and impaired lipid metabolism in aged rats. J Lipid Res 2004;45:729-35.
[189] Hagen TM, Moreau R, Suh JH, Visioli F. Mitochondrial decay in the aging rat heart: evidence for improvement by dietary supplementation with acetyl-L-carnitine and/or lipoic acid. Ann NY Acad Sci 2002;959:491-507.
[190] Kim JH, Shrago E, Elson CE. Age-related changes in respiration coupled to phosphorylation. II. Cardiac mitochondria. Mech Ageing Dev 1988;46:279-90.

[191] Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci USA 1994;91:10771-8.
[192] Hagen TM, Wehr CM, Ames BN. Mitochondrial decay in aging. Reversal through supplementation of acetyl-L-carnitine and N-tert-butyl-alpha-phenyl-nitrone. Ann N Y Acad Sci 1998;854:214-23.
[193] Amicarelli F, Ragnelli AM, Aimola P, Bonfigli A, Colafarina S, Di Ilio C, et al. Age-dependent ultrastructural alterations and biochemical response of rat skeletal muscle after hypoxic or hyperoxic treatments. Biochim Biophys Acta 1999;1453:105-14.
[194] de Cavanagh EM, Piotrkowski B, Basso N, Stella I, Inserra F, Ferder L, et al. Enalapril and losartan attenuate mitochondrial dysfunction in aged rats. FASEB J 2003;17:1096-8.
[195] Yasuda K, Ishii T, Suda H, Akatsuka A, Hartman PS, Goto S, et al. Age-related changes of mitochondrial structure and function in Caenorhabditis elegans. Mech Ageing Dev 2006;127:763-70.
[196] Choksi KB, Nuss JE, Boylston WH, Rabek JP, Papaconstantinou J. Age-related increases in oxidatively damaged proteins of mouse kidney mitochondrial electron transport chain complexes. Free Radic Biol Med 2007;43:1423-38.
[197] Barth PG, Scholte HR, Berden JA, Van der Klei-Van Moorsel JM, Luyt-Houwen IE, Van 't Veer-Korthof ET, et al. An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leucocytes. J Neurol Sci 1983;62:327-55.
[198] Barth PG, Wanders RJ, Vreken P, Janssen EA, Lam J, Baas F. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome) (MIM 302060). J Inherit Metab Dis 1999;22:555-67.
[199] Bentlage HA, Wendel U, Schagger H, ter Laak HJ, Janssen AJ, Trijbels JM. Lethal infantile mitochondrial disease with isolated complex I deficiency in fibroblasts but with combined complex I and IV deficiencies in muscle. Neurology 1996;47:243-8.
[200] Figarella-Branger D, Pellissier JF, Scheiner C, Wernert F, Desnuelle C. Defects of the mitochondrial respiratory chain complexes in three pediatric cases with hypotonia and cardiac involvement. J Neurol Sci 1992;108:105-13.
[201] Pitkanen S, Robinson BH. Mitochondrial complex I deficiency leads to increased production of superoxide radicals and induction of superoxide dismutase. J Clin Invest 1996;98:345-51.
[202] Tiranti V, Hoertnagel K, Carrozzo R, Galimberti C, Munaro M, Granatiero M, et al. Mutations of SURF-1 in Leigh disease

associated with cytochrome c oxidase deficiency. Am J Hum Genet 1998;63:1609-21.

[203] Dudkina NV, Eubel H, Keegstra W, Boekema EJ, Braun HP. Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. Proc Natl Acad Sci USA 2005;102:3225-9.

[204] Dudkina NV, Sunderhaus S, Boekema EJ, Braun HP. The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. J Bioenerg Biomembr 2008;40:419-24.

[205] Vonck J, Schafer E. Supramolecular organization of protein complexes in the mitochondrial inner membrane. Biochim Biophys Acta 2009;1793:117-24.

[206] Schafer E, Dencher NA, Vonck J, Parcej DN. Three-dimensional structure of the respiratory chain supercomplex I1III2IV1 from bovine heart mitochondria. Biochemistry 2007;46:12579-85.

[207] Hagen TM, Moreau R, Suh JH, Visioli F. Mitochondrial decay in the aging rat heart: evidence for improvement by dietary supplementation with acetyl-L-carnitine and/or lipoic acid. Ann N Y Acad Sci 2002;959:491-507.

[208] Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. J Mol Cell Cardiol 2001;33:1065-89.

[209] Palmer JW, Tandler B, Hoppel CL. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. J Biol Chem 1977;252:8731-9.

[210] Wittig I, Braun HP, Schagger H. Blue native PAGE. Nat Protoc 2006;1:418-28.

[211] Wittig I, Schagger H. Electrophoretic methods to isolate protein complexes from mitochondria. Methods Cell Biol 2007;80:723-41.

[212] Fandiño AS, Rais I, Vollmer M, Elgass H, Schagger H, Karas M. LC-nanospray-MS/MS analysis of hydrophobic proteins from membrane protein complexes isolated by blue-native electrophoresis. J Mass Spectrom 2005;40:1223-31.

[213] Schagger H, Pfeiffer K. The ratio of oxidative phosphorylation complexes I-V in bovine heart mitochondria and the composition of respiratory chain supercomplexes. J Biol Chem 2001;276:37861-7.

[214] Zhang M, Mileykovskaya E, Dowhan W. Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation

in the inner mitochondrial membrane. J Biol Chem 2002;277:43553-6.

[215] Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, et al. Cardiolipin stabilizes respiratory chain supercomplexes. J Biol Chem 2003;278:52873-80.

[216] Nijtmans LG, de Jong L, Artal Sanz M, Coates PJ, Berden JA, Back JW, et al. Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. EMBO J 2000;19:2444-51.

[217] Coates PJ, Nenutil R, McGregor A, Picksley SM, Crouch DH, Hall PA, et al. Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. Exp Cell Res 2001;265:262-73.

[218] Piper PW, Jones GW, Bringloe D, Harris N, MacLean M, Mollapour M. The shortened replicative life span of prohibitin mutants of yeast appears to be due to defective mitochondrial segregation in old mother cells. Aging cell 2002;1:149-57.

[219] Merkwirth C, Langer T. Prohibitin function within mitochondria: essential roles for cell proliferation and cristae morphogenesis. Biochim Biophys Acta 2009;1793:27-32.

[220] Lombardi A, Silvestri E, Cioffi F, Senese R, Lanni A, Goglia F, et al. Defining the transcriptomic and proteomic profiles of rat ageing skeletal muscle by the use of a cDNA array, 2D- and Blue native-PAGE approach. J Proteomics 2009;72:708-21.

[221] Wenz T, Hielscher R, Hellwig P, Schagger H, Richers S, Hunte C. Role of phospholipids in respiratory cytochrome bc(1) complex catalysis and supercomplex formation. Biochim Biophys Acta 2009;1787:609-16.

[222] Schlame M, Ren M. The role of cardiolipin in the structural organization of mitochondrial membranes. Biochim Biophys Acta 2009;1788:2080-3.

[223] Schlame M, Horvath L, Vigh L. Relationship between lipid saturation and lipid-protein interaction in liver mitochondria modified by catalytic hydrogenation with reference to cardiolipin molecular species. Biochem J 1990;265:79-85.

[224] Diaz F, Enriquez JA, Moraes CT. Cells Lacking Rieske Iron-Sulfur Protein Have a Reactive Oxygen Species-Associated Decrease in Respiratory Complexes I and IV. Mol Cell Biol 2012;32:415-29.

[225] Vukotic M, Oeljeklaus S, Wiese S, Vogtle FN, Meisinger C, Meyer HE, et al. Rcf1 mediates cytochrome oxidase assembly and

respirasome formation, revealing heterogeneity of the enzyme complex. Cell Metab 2012;15:336-47.

[226] Strogolova V, Furness A, Robb-McGrath M, Garlich J, Stuart RA. Rcf1 and Rcf2, Members of the Hypoxia-Induced Gene 1 Protein Family, Are Critical Components of the Mitochondrial Cytochrome bc1-Cytochrome c Oxidase Supercomplex. Mol Cell Biol 2012;32:1363-73.

[227] Koshkin V, Greenberg ML. Oxidative phosphorylation in cardiolipin-lacking yeast mitochondria. Biochem J 2000;347 Pt 3:687-91.

[228] Koshkin V, Greenberg ML. Cardiolipin prevents rate-dependent uncoupling and provides osmotic stability in yeast mitochondria. Biochem J 2002;364:317-22.

[229] Jiang F, Rizavi HS, Greenberg ML. Cardiolipin is not essential for the growth of Saccharomyces cerevisiae on fermentable or non-fermentable carbon sources. Mol Microbiol 1997;26:481-91.

[230] Panov A, Dikalov S, Shalbuyeva N, Hemendinger R, Greenamyre JT, Rosenfeld J. Species- and tissue-specific relationships between mitochondrial permeability transition and generation of ROS in brain and liver mitochondria of rats and mice. Am J Physiol Cell Physiol 2007;292:C708-18.

[231] Riva A, Tandler B, Loffredo F, Vazquez E, Hoppel C. Structural differences in two biochemically defined populations of cardiac mitochondria. Am J Physiol Heart Circ Physiol 2005;289:H868-72.

[232] Kaasik A, Veksler V, Boehm E, Novotova M, Minajeva A, Ventura-Clapier R. Energetic crosstalk between organelles: architectural integration of energy production and utilization. Circ Res 2001;89:153-9.

[233] Sasaki N, Sato T, Marban E, O'Rourke B. ATP consumption by uncoupled mitochondria activates sarcolemmal K(ATP) channels in cardiac myocytes. Am J Physiol Heart Circ Physiol 2001;280:H1882-8.

[234] Hoppel CL, Tandler B, Parland W, Turkaly JS, Albers LD. Hamster cardiomyopathy. A defect in oxidative phosphorylation in the cardiac interfibrillar mitochondria. J Biol Chem 1982;257:1540-8.

[235] Schlame M, Kelley RI, Feigenbaum A, Towbin JA, Heerdt PM, Schieble T, et al. Phospholipid abnormalities in children with Barth syndrome. J Am Coll Cardiol 2003;42:1994-9.

[236] Vreken P, Valianpour F, Nijtmans LG, Grivell LA, Plecko B, Wanders RJ, et al. Defective remodeling of cardiolipin and

phosphatidylglycerol in Barth syndrome. Biochem Biophys Res Commun 2000;279:378-82.

[237] Brandner K, Mick DU, Frazier AE, Taylor RD, Meisinger C, Rehling P. Taz1, an outer mitochondrial membrane protein, affects stability and assembly of inner membrane protein complexes: implications for Barth Syndrome. Molecular biology of the cell 2005;16:5202-14.

[238] Acehan D, Vaz F, Houtkooper RH, James J, Moore V, Tokunaga C, et al. Cardiac and skeletal muscle defects in a mouse model of human Barth syndrome. J Biol Chem 2011;286:899-908.

[239] van Gestel RA, Rijken PJ, Surinova S, O'Flaherty M, Heck AJ, Killian JA, et al. The influence of the acyl chain composition of cardiolipin on the stability of mitochondrial complexes; an unexpected effect of cardiolipin in alpha-ketoglutarate dehydrogenase and prohibitin complexes. J Proteomics 2010;73:806-14.

[240] Kolarovic L, Fournier NC. A comparison of extraction methods for the isolation of phospholipids from biological sources. Anal Biochem 1986;156:244-50.

[241] An HJ, Shin H, Jo SG, Kim YJ, Lee JO, Paik SG, et al. The survival effect of mitochondrial Higd-1a is associated with suppression of cytochrome C release and prevention of caspase activation. Biochim Biophys Acta 2011;1813:2088-98.

[242] Zhang M, Mileykovskaya E, Dowhan W. Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. J Biol Chem 2005;280:29403-8.

[243] Houtkooper RH, Rodenburg RJ, Thiels C, van Lenthe H, Stet F, Poll-The BT, et al. Cardiolipin and monolysocardiolipin analysis in fibroblasts, lymphocytes, and tissues using high-performance liquid chromatography-mass spectrometry as a diagnostic test for Barth syndrome. Anal Biochem 2009;387:230-7.

[244] Davila-Roman VG, Vedala G, Herrero P, de las Fuentes L, Rogers JG, Kelly DP, et al. Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. J Am Coll Cardiol 2002;40:271-7.

[245] Lesnefsky EJ, Gudz TI, Migita CT, Ikeda-Saito M, Hassan MO, Turkaly PJ, et al. Ischemic injury to mitochondrial electron transport in the aging heart: damage to the iron-sulfur protein subunit of electron transport complex III. Arch Biochem Biophys 2001;385:117-28.

[246] Grantham BD, Zammit VA. Restoration of the properties of carnitine palmitoyltransferase I in liver mitochondria during re-feeding of starved rats. Biochem J 1986;239:485-8.
[247] Grantham BD, Zammit VA. Role of carnitine palmitoyltransferase I in the regulation of hepatic ketogenesis during the onset and reversal of chronic diabetes. Biochem J 1988;249:409-14.
[248] Takahashi M, Ueda S, Misaki H, Sugiyama N, Matsumoto K, Matsuo N, et al. Carnitine determination by an enzymatic cycling method with carnitine dehydrogenase. Clin Chem 1994;40:817-21.
[249] Nakamura T, Sugihara H, Kinoshita N, Ito K, Adachi Y, Hirasaki S, et al. Serum carnitine concentrations in patients with idiopathic hypertrophic cardiomyopathy: relationship with impaired myocardial fatty acid metabolism. Clin Sci 1999;97:493-501.
[250] Moreau R, Heath SH, Doneanu CE, Harris RA, Hagen TM. Age-related compensatory activation of pyruvate dehydrogenase complex in rat heart. Biochem Biophys Res Commun 2004;325:48-58.
[251] Gobin S, Thuillier L, Jogl G, Faye A, Tong L, Chi M, et al. Functional and structural basis of carnitine palmitoyltransferase 1A deficiency. J Biol Chem 2003;278:50428-34.
[252] Morillas M, Lopez-Vinas E, Valencia A, Serra D, Gomez-Puertas P, Hegardt FG, et al. Structural model of carnitine palmitoyltransferase I based on the carnitine acetyltransferase crystal. Biochem J 2004;379:777-84.
[253] Jogl G, Hsiao YS, Tong L. Crystal structure of mouse carnitine octanoyltransferase and molecular determinants of substrate selectivity. J Biol Chem 2005;280:738-44.
[254] Sharma V, Abraham T, So A, Allard MF, McNeill JH. Functional effects of protein kinases and peroxynitrite on cardiac carnitine palmitoyltransferase-1 in isolated mitochondria. Mol Cell Biochem 2010;337:223-37.
[255] Pesce V, Fracasso F, Cassano P, Lezza AM, Cantatore P, Gadaleta MN. Acetyl-L-carnitine supplementation to old rats partially reverts the age-related mitochondrial decay of soleus muscle by activating peroxisome proliferator-activated receptor gamma coactivator-1alpha-dependent mitochondrial biogenesis. Rejuvenation Res 2010;13:148-51.
[256] Liu J, Head E, Gharib AM, Yuan W, Ingersoll RT, Hagen TM, et al. Memory loss in old rats is associated with brain mitochondrial decay and RNA/DNA oxidation: partial reversal by feeding acetyl-L-

carnitine and/or R-alpha -lipoic acid. Proc Natl Acad Sci USA 2002;99:2356-61.

[257] Costell M, Grisolia S. Effect of carnitine feeding on the levels of heart and skeletal muscle carnitine of elderly mice. FEBS Lett 1993;315:43-6.

[258] Musicco C, Capelli V, Pesce V, Timperio AM, Calvani M, Mosconi L, et al. Rat liver mitochondrial proteome: Changes associated with aging and acetyl-L-carnitine treatment. J Proteomics 2011;74:2536-47.

[259] Rump TJ, Abdul Muneer PM, Szlachetka AM, Lamb A, Haorei C, Alikunju S, et al. Acetyl-L-carnitine protects neuronal function from alcohol-induced oxidative damage in the brain. Free Radic Biol Med 2010;49:1494-504.

[260] Cui J, Das DK, Bertelli A, Tosaki A. Effects of L-carnitine and its derivatives on postischemic cardiac function, ventricular fibrillation and necrotic and apoptotic cardiomyocyte death in isolated rat hearts. Mol Cell Biochem 2003;254:227-34.

[261] Calvani M, Reda E, Arrigoni-Martelli E. Regulation by carnitine of myocardial fatty acid and carbohydrate metabolism under normal and pathological conditions. Basic Res Cardiol 2000;95:75-83.

[262] Poon HF, Calabrese V, Calvani M, Butterfield DA. Proteomics analyses of specific protein oxidation and protein expression in aged rat brain and its modulation by L-acetylcarnitine: insights into the mechanisms of action of this proposed therapeutic agent for CNS disorders associated with oxidative stress. Antioxid Redox Signal 2006;8:381-94.

[263] Cassano P, Sciancalepore AG, Pesce V, Fluck M, Hoppeler H, Calvani M, et al. Acetyl-L-carnitine feeding to unloaded rats triggers in soleus muscle the coordinated expression of genes involved in mitochondrial biogenesis. Biochim Biophys Acta 2006;1757:1421-8.

[264] Calabrese V, Cornelius C, Dinkova-Kostova AT, Iavicoli I, Di Paola R, Koverech A, et al. Cellular stress responses, hormetic phytochemicals and vitagenes in aging and longevity. Biochim Biophys Acta 2012;1822:753-83.

[265] Calabrese V, Cornelius C, Stella AM, Calabrese EJ. Cellular stress responses, mitostress and carnitine insufficiencies as critical determinants in aging and neurodegenerative disorders: role of hormesis and vitagenes. Neurochem Res 2010;35:1880-915.

[266] Calo LA, Pagnin E, Davis PA, Semplicini A, Nicolai R, Calvani M, et al. Antioxidant effect of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. Int J Cardiol 2006;107:54-60.
[267] Mansour HH. Protective role of carnitine ester against radiation-induced oxidative stress in rats. Pharmacol Res 2006;54:165-71.
[268] Judge S, Leeuwenburgh C. Cardiac mitochondrial bioenergetics, oxidative stress, and aging. Am J Physiol Cell Physiol 2007;292:C1983-92.