

# AN ABSTRACT OF THE DISSERTATION OF

Jung Hyuk Suh for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on July 15, 2003.

## Antioxidant Mechanisms of Ascorbate and (R)-Alpha-Lipoic Acid in Aging and Transition Metal Ion-Mediated Oxidative Stress

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Oxidative stress is the major driving force behind the aging process and many age-related diseases. However, direct experimental evidence of whether antioxidants, such as ascorbate (AA) and lipoic acid (LA) can slow the progression of aging process and/or reduce risks of developing degenerative disease is largely absent. This suggests a better understanding of the precise mechanism of how dietary micronutrient affect parameters of involved in cellular redox balance and aging are warranted. In this dissertation, young and old rats were used as our model to understand potential pro-oxidant events that contribute to increases in oxidative stress in various tissues and how antioxidants such as ascorbate and lipoic acid influence these events. Our major findings are that the age-related impairment of mitochondria and increased deposition of iron contribute significantly to heighten levels of oxidative stress, as evidenced by

the resultant increases in the rates of oxidant appearance and in the levels of oxidative damage to DNA, lipids and proteins. We find that AA and LA strongly protected against transition metal-ion dependent increases in oxidative stress. AA effectively inhibited transition metal-mediated lipide peroxidation in human plasma. LA in its reduced form effectively binds iron and copper in a redox inactive manner and reversed chronically elevated levels of iron in the brain without removing enzyme bound transition metal ions. LA also significantly attenuated the age-related increase in oxidative stress associated with mitochondrial decay in the heart, as evidenced by the improvements in AA levels and glutathione redox status. The declines in tissue GSH levels in aged rats were strongly associated with the diminished  $\gamma$ -GCL activity (in parallel with decreased expression of the catalytic and modulatory subunits), and lowered Nrf2 expression and binding to ARE sequence in rat liver. Remarkably, all these events were effectively reversed by the administration of LA, modulating the parameters to return to the observed in young animals. The implications of this work open new avenues not only for further understanding of the aging process but also for possible strategies in its modulation by the micronutrients.

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**Antioxidant Mechanisms of Ascorbate and (*R*)-Alpha-Lipoic Acid in  
Aging and Transition Metal Ion-Mediated Oxidative Stress**

by

**Jung Hyuk Suh**

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Jung Hyuk Suh, Author

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**“Discovery consists in seeing what everybody else has seen and thinking what nobody has thought.”**

**Albert Szent-Gyorgyi**

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## CONTRIBUTION OF AUTHORS

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**Antioxidant Mechanisms of Ascorbate and (R)-Alpha-Lipoic Acid  
in Aging and Transition Metal Ion-Mediated Oxidative Stress**

**Chapter 1**

**General Introduction**

## 1.1 General Introduction

The aging process is defined by the progressive accumulation of changes that lead to compromised physiological functions and increased susceptibility to disease and death [1]. The mechanisms underlying the aging process are undoubtedly complex and are influenced by both genetic and epigenetic factors such as diet and environment exposures. Moreover, the inherent difficulties in dissociating the effects of aging from the effects of age-related pathologies makes it extremely challenging to define exact cellular events that contribute to the aging process. Despite the multifaceted nature of aging process, it now appears that oxidative stress play a central role in the aging process.

One of the most widely accepted theory of aging is the “free radical theory of aging”, initially proposed in 1956 by Denham Harman. According to this theory, the gradual accumulation of damage to cellular constituents mediated by free radicals (physiologically produced as by-products of normal aerobic metabolism) are causally involved in the aging process [1]. This definition emphasizes that oxidants (referring to both reactive oxygen and nitrogen species) produced endogenously act as a deleterious agents that cause stochastic damage, leading to impairment of function and death. The validity of free radical theory of aging has been rigorously tested and evidence strongly supports the central role for oxidative stress in the aging process. For instance, studies show a strong inverse correlation between species-specific rates

of mitochondrial superoxide generation and their maximal life [2,3]. Similar inverse correlation between the maximal life span and the steady state levels of oxidative damage to mitochondrial deoxyribonucleic acid (DNA) can also be observed across different species [4,5]. These studies clearly demonstrate that generation of reactive oxygen species and oxidative damage are inherent part of the aging process.

### *Oxygen toxicity*

Spontaneous oxidation of biological molecules does not occur readily, mainly because of the unique electron distribution of the ground state dioxygen. Oxygen has two unpaired electrons, each located in different Pi orbitals, but having parallel spins [6]. Any molecule donating a pair of electrons to oxygen must have electrons that have opposite spins from the electrons present in oxygen. The requirement for spin inversion prohibits most biological molecules from directly reacting with oxygen [7].

Because of this spin restriction, oxygen preferentially accepts single electrons at a time from other radicals. Thus, enzymes that carry out two or four electron reductions of oxygen *in vivo* possess transition metals (such as iron or copper) in their active sites to facilitate sequential one-electron reduction steps [8]. Sequential one and two electron reduction of oxygen leads to generation of superoxide ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ), respectively. Hydrogen peroxide can further undergo a one-electron reduction, which also facilitated by transition metals, to yield the highly

reactive hydroxyl radical ( $\text{HO}^\bullet$ ) [8]. It is clear that oxidants are generated *in vivo* and cause significant damage to cellular macromolecules [9,10].

### *Mitochondria as the major intracellular source of oxidants*

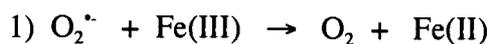
Mitochondria are generally considered to be the most significant source of  $\text{O}_2^\bullet$  and  $\text{H}_2\text{O}_2$  in cells [11]. This is because mitochondrial oxygen consumption account for approximately 90% of total cellular oxygen consumption [12]. It has been shown that during State 4 respiration mitochondria generate  $\text{O}_2^\bullet$  and  $\text{H}_2\text{O}_2$  at levels which amount to about 1-2% of total mitochondrial oxygen uptake [13]. Mitochondrial oxidant generation is due to the partial reduction of oxygen by electrons that leaks from unstable ubiquinone, or semiquinone anion that are from during redox cycling of ubiquinone (Q cycle) present in Complex III [14]. In addition to sites in Complex III, Complex I of the mitochondrial electron transport chain produce  $\text{O}_2^\bullet$  during the reoxidation flavin mononucleotide (FMN) of NADH-ubiquinone reductase [15] and turn over of iron-sulfur centers [15].

Previous studies examining the age-related changes in mitochondria isolated from old animals have reported that mitochondrial oxidant production increases with age [16]. However, a study by Hansford and co-workers suggested that the increase in mitochondrial oxidant production observed with age may be attributed to an artifact stemming from assay conditions due to excessively high levels of substrate (succinate) used [17]. In addition, mitochondria in muscles such as the heart are heterogeneous in

nature and appear to be distributed into two localized groups. Subsarcolemmal mitochondria (SSM) are adjacent to the subsarcolemmal membrane while interfibrillary mitochondria (IFM) are tightly associated within myofibrils [18]. However, the exact nature and the extent of potential age-related changes in these two sub-populations of mitochondria are not completely clear.

### *Pro-oxidant roles of transition metal-ions*

In many *in vitro* systems, neither  $O_2^{\cdot -}$  nor  $H_2O$  alone can initiate lipid peroxidation in absence of transition metal ions [19]. The chemistry of iron or copper-mediated free radical production and toxicity can be explained by the Haber-Weiss reaction, which is summarized as follows [19]:



In the first step of the reaction  $O_2^{\cdot -}$  reduces, in this case, iron (reaction 1), which subsequently reacts with hydrogen peroxide produced by  $O_2^{\cdot -}$  dismutation (reaction 2). The final step of this reaction sequence, also referred to as the Fenton reaction, leads to the generation of  $HO^{\cdot}$  [6].

The problem of iron toxicity is largely circumvented by tightly regulating the availability of free, redox active iron. In extracellular fluids, such as plasma and cerebrospinal fluids, the transferrin (79 kDa) protein binds 2 Fe(III) molecules. Iron

bound to transferrin does not catalyze hydroxyl radical formation [8]. The main iron storage protein in cells is ferritin (480 kDa). Ferritin can bind up to 4500 atoms of iron per one molecule of ferritin. Iron (Fe(III)) bound to ferritin is not available for Fenton reaction but its exposure to superoxide can release it [20]. Both ferritin and transferrin are only partially saturated with iron. In addition to transferrin and ferritin, other proteins, such as haemosiderin and lactoferrin, assist in binding free iron or heme molecules.

In most cells, cellular iron level is primarily regulated by coordinated expression of the transferrin receptor (TfR) and ferritin. The rate of TfR and ferritin synthesis is primarily regulated post-transcriptionally level by the cytosolic protein called iron regulatory protein (IRP) [21]. When intracellular iron levels are sufficient, IRP acts as a cytosolic aconitase, which contains 4 Fe/4sulfur cluster in the active site. Once intracellular iron levels decreased sufficiently, there is a disassembly of these iron-sulfur clusters which allows IRP to bind to a specific stem loop sequence in the mRNA of target proteins identified as the iron regulatory element (IRE). The binding of IRP to the IRE located in the 3'-untranslated region (UTR) of TfR message increases the stability of its mRNA. Conversely, IRP-binding to the IRE in the 5'-UTR of ferritin inhibits ferritin translation. Thus, activation of IRP leads to increased synthesis of TfR and the inhibition of ferritin translation, leading to increased iron uptake and restoration of depleted intracellular iron pools.

Despite this tight regulation, iron has been shown to accumulate significantly with age in tissues such as the brain. Age-related increase in iron levels far exceeds the amount of iron needed to maintain normal function [22]. Post mortem analysis of

normal aged subjects reveal that the brain iron content in the basal ganglia can increase to levels as high as 200  $\mu\text{g/g}$  tissue (compared to liver iron content of  $\sim 150$   $\mu\text{g/g}$  tissue) [23]. The observed increase in cytosolic iron levels may be a reflection of deposition of aging pigments, such as lipofuscin [24].

Lipofuscin is a complex, insoluble intracellular pigment that is characterized to contain polymerized residues of oxidized lipids and proteins [25]. In experimental animals, lipofuscin has been shown to increase linearly with age in neurons throughout the central nervous system [24-27]. In addition, there is also a progressive accumulation of neuromelanin with aging [28].

Neuromelanin is characterized by deposition of polymerized dopamine oxidation products. In aging, neuromelanin has been shown to accumulate in the brain stem nuclei (substantia nigra, locus coeruleus, and dorsal motor nucleus of vagus nerve) [28]. Neuromelanin has high binding affinity for iron and has been demonstrated to be the major iron storage protein in the substantia nigra region of the brain [29]. Under normal conditions, the chelation of iron by neuromelanin may be protective but during conditions of iron overload, the iron binding sites may become saturated, leading to increased availability of free redox active iron. The saturation of iron loosely bound to "aging pigments" may potentially increase oxidant load and result in the observed increased rate of antioxidant consumption.

The disruption in iron homeostasis in aged brain is likely to have a profound impact in the brain since it contains relatively low levels of iron binding proteins, such as transferrin [7,30]. An obvious consequence of such dramatic changes in tissue iron

levels may be increased oxidative stress. Under these pathophysiological conditions, it is not clear whether antioxidants such as ascorbate or lipoic acid have any benefit in modulating oxidative stress in the brain.

### *Modulation of oxidative stress by antioxidant supplementation*

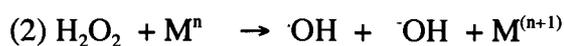
Oxidative stress, as defined by the imbalance between pro- and antioxidant processes in favor of the former, is believed to be the major driving force behind the aging process and in the initiation of many chronic age-related diseases [31-33]. A corollary to this view is that increasing the dietary intake of specific micronutrients and antioxidants should be effective in delaying aging and preventing age-related illnesses. However, there is no conclusive evidence that megadoses of antioxidants has any benefit in preventing the pathogenesis of diseases and/or in improving health in the elderly [34]. In a primary intervention trial, dietary antioxidants, such as  $\beta$ -carotene, has even been found to promote, rather than prevent, incidence of lung cancer [35]. This study illustrates the need for a more precise biochemical understanding of dietary micronutrients that act as antioxidant.

### *Potential pro-oxidant role of ascorbate*

Ascorbate is very effect chain terminating antioxidant for a number of different reasons. Thermodynamically, ascorbate has very low one-electron reduction potential

which allows it to react with virtually all physiologically relevant radicals [36]. At the same time, its one-electron oxidation product, the ascorbyl radical, is relatively harmless due to its low reactivity. Furthermore, the ascorbyl radical has a very slow reactivity with oxygen and it is highly unlikely that the autoxidation of ascorbyl radicals lead to the production of superoxide. The efficiency of ascorbate as an antioxidant is also enhanced by the fact that ascorbate can be recycled from dehydroascorbate by a number of enzyme systems that use NADH or NADPH as reducing equivalents [37]. Ascorbate has been shown to be an effective scavenger of various radical species, such as hydroxyl radicals, superoxide, peroxy radicals, reactive nitrogen species such as peroxynitrite (ONOO<sup>-</sup>) and hypochlorous acid (HOCl) [38-40].

Ascorbate, in addition to its ability to act as an antioxidant, can also exhibit pro-oxidant properties in the presence of iron *in vitro* [41]. Because of its reducing capabilities, ascorbate added to a Fenton system can catalyze reactions that produce hydroxyl radicals. The reduced metal ions can also catalyze the production of alkoxy radicals from lipid hydroperoxide, which can initiate and propagate lipid peroxidation. These reactions are summarized as followed:



Reaction (1): Reduction of redox active metal ions by ascorbate to form ascorbyl radical and the reduced metal ion.

Reaction (2): Production of highly reactive hydroxyl radicals from the reaction of hydrogen peroxide with the reduced metal ion.

Reaction (3): Reaction of lipid hydroperoxides with reduced metal ions to form alkoxy radicals.

Alternatively, ascorbate can also interact with iron to form either a perferryl iron complex or a ferryl complex that has an oxidizing potential comparable to hydroxyl radical [19]. In this model, ascorbate would become pro-oxidant only when it maintains the 1:1 stoichiometric ratio of Fe(II):Fe(III). At higher concentrations of ascorbate, the complete reduction to Fe(II) state will inhibit lipid peroxidation.

Based on these *in vitro* characterizations of ascorbate, it is possible that ascorbate may potentially exacerbate transition metal mediated oxidative stress. Given the potential deleterious effects the age-related deposition of transition metals, there is a clear need to understand whether ascorbate positively or negatively effects transition metal catalyzed oxidant generation. However, to date, the exact physiological relevance of the putative pro-oxidant role of ascorbate has not been fully examined. Thus, it is still controversial as to how antioxidants influence oxidative stress parameters in certain aging tissues, such as the brain.

*(R)- $\alpha$ -lipoic acid as a metabolic antioxidant*

(*R*)- $\alpha$ -lipoic acid (LA) is a thiol compound found naturally in plants and animals [42]. Lipoamide dehydrogenases, found only in mitochondria, reduce free LA to dihydrolipoic acid (DHLA), which is a potent antioxidant. Thus, LA supplementation may increase cellular and mitochondrial antioxidant status, thereby effectively attenuating any putative increase in oxidative stress with age [43].

Aside from acting as a potent antioxidant in its own right, LA increases or maintains levels of other low molecular weight antioxidants, particularly glutathione (GSH) and ascorbic acid (AA). LA may exert these effects by “sparing” both GSH and vitamin C [44] or, in the case of GSH, by increasing the cellular uptake of cysteine [45], which is the rate limiting substrate for GSH biosynthesis. Given its multifaceted roles as a metabolic co-factor and an antioxidant, LA appears to be an ideal agent that may attenuate the age-related increase in oxidative stress.

*Metabolic role of (R)- $\alpha$ -lipoic acid*

In eukaryotic cells, lipoic acid is covalently attached to  $\epsilon$ -nitrogen of lysyl residues via an amide linkage. There are five known enzymes associated with lipoic acid [42]. Four of these proteins are found in the three  $\alpha$ -keto acid dehydrogenase complexes, namely the pyruvate dehydrogenase complex, the  $\alpha$ -ketoglutarate dehydrogenase complex, and the branched chain keto acid dehydrogenase complex

[46]. The fifth lipoamide moiety is found in the glycine cleavage system. Typically,  $\alpha$ -keto acid dehydrogenases in mitochondria consist of multiple copies of three enzymes, identified as E1 ( $\alpha$ -keto acid dehydrogenase), E2 (dihydrolipoyl acyltransferase), and E3 (dihydrolipoyl dehydrogenase) [47]. These three enzymes catalyze five reactions that oxidatively decarboxylate their substrates. The main role of lipoamide in this process is to shuttle the acyl group from thiamine pyrophosphate of E1 to coenzyme A to produce acyl-CoA. During this process, lipoamide is reduced and E3 reoxidizes the lipoamide for another cycle using  $\text{NAD}^+$  as an electron acceptor. Lipoamide has also been shown to associate with protein X and has no apparent catalytic activity but may aid in structural binding of the E2 domain to E3 [46].

#### *(R)- $\alpha$ -lipoic acid as an antioxidant*

*(R)- $\alpha$ -lipoic acid* is a potent thiol antioxidant. The reduction potential of dihydrolipoic acid (DHLA)/lipoic acid redox couple is very low ( $-320$  mV) which makes it one of the most potent cellular reductant, surpassed only by NADH or NADPH [48]. *In vitro*, both lipoic acid and DHLA has shown to be a potent scavenger of hydroxyl radicals, hypochlorous acid, singlet oxygen and nitric oxide [49-51]. It is interesting to note that unlike other disulfides, oxidized lipoic acid also shows potent scavenging capacity. The greater reactivity of lipoic acid compared to

other disulfide (i.e., GSSG) may be due the strained conformation of the five-membered bithiolane ring of lipoic acid [52].

### *Metal chelation by Lipoic Acid*

Various *in vitro* experiments show that both lipoic acid and DHLA chelates metal-ions. Lipoic acid and its chain shortened catabolites such as binorlipoate and tetranorlipoate can form stable complexes with divalent metals such as Mn(II), Cu(II) and Zn(II) in solution [53]. Both dithiolane and carboxylate groups on lipoic acid can form stable complexes with metal ions in solution. The shorter chain catabolites of lipoic acid showed stronger affinity for divalent metals possibly due to increased participation of the dithiolane functional moiety [53]. Lipoic acid (100  $\mu$ M) was also found to inhibit Cu(II) (0.2  $\mu$ M) induced ascorbate oxidation *in vitro*, suggesting that the resulting complex between Cu(II) and  $\alpha$ -lipoic acid was somewhat redox inactive. Lipoic acid also increased the partitioning of Cu(II) into n-octanol from aqueous solution and inhibited Cu(II) induced liposomal oxidation [54].

Study examining DHLA effect on Cu(II) dependent low-density lipoprotein (LDL) oxidation show that DHLA inhibited Cu(II) mediated LDL oxidation by chelating the copper through its thiol groups. The stability of Cu(II):DHLA was found to be most stable at low pH (< 6). At low pH, carboxylate group of  $\alpha$ -lipoic acid would be protonated and chelation would be most dependent of the dithiol moiety. Thus suggesting that thiol groups in DHLA may form a more stable complex than the

complex coordinated by carboxylate group [55]. The evidence for  $\alpha$ -lipoic acid chelation of Fe(II) is much less conclusive. One study by Scott and co-workers reported that  $\alpha$ -lipoic acid inhibited the site-specific degradation by  $\text{FeCl}_3/\text{H}_2\text{O}_2$ /ascorbate system possibly by removing iron from deoxyribose [56]. In contrast, Bast and Haenen reported that lipoic acid was unable to inhibit  $\text{Fe}^{3+}\text{SO}_4$  mediated lipid oxidation in rat liver microsomal system [57]. Another study, however found that DHLA under anaerobic conditions was able to remove iron from ferritin. In this study, DHLA (concentration range of 1.12 mM to 5.5 mM) was able to remove ferritin bound iron over a wide range of pH (pH 5.5 – 9) [58]. The removal of iron by DHLA was most effective at alkaline pH suggesting that thiolate species was likely to be involved in the process.

Given the significant contributions of mitochondrial decay, and transition metals accumulation to the age-related increases in oxidative stress, new innovative strategies that can ameliorate these pro-oxidant influences may be needed. In this regard, dietary antioxidants such as ascorbate and lipoic acid may elicit a multitude of physiological effects in modulating cellular metabolism, antioxidant status and metal toxicity. However, their potential application to alleviate age-associated increase in oxidative stress has not been thoroughly examined.

Thus, the major aims of this dissertation were to examine the effects of two well-known dietary antioxidants, namely ascorbate (AA) and lipoic acid (LA), on pro-oxidant events associated with aging. Specifically, we addressed whether ascorbate and lipoic acid (LA) protect against the toxicity mediated by transition metal-ions that

have been shown to accumulate with age. Furthermore, we sought to define how age-related changes in mitochondrial function modulate cellular and mitochondrial antioxidant and thiol redox status and whether dietary supplementation with LA can reverse these changes. Finally, we examined the mechanisms underlying LA-mediated improvements in age-related declines in cellular glutathione status.

The specific aims addressed in this dissertation are as follows:

**1.) To determine the physiological relevance of the potential pro-oxidant interactions between transition metal ions (iron and copper) and ascorbate.**

To address this aim, we used human plasma as a physiological relevant buffer to determine the effects of varying concentrations of ascorbate against iron- and copper-mediated oxidation of plasma lipids and proteins (chapter 2).

**2.) To characterize the transition metal-chelating abilities of lipoic acid and dihydrolipoic acid.**

LA and DHLA have been shown to chelate metal ions *in vitro*, however, it is not known whether they bind transition metals in a redox inactive form or whether dietary supplement of LA may be able to lower the age-related accumulation of iron in aging tissues. To address this issue, we determined the chelating properties of LA by addressing three specific subaims: 2.1) to examine whether LA and DHLA bind transition metals in redox inactive form, 2.2) to determine whether LA and DHLA remove metals from metalloenzymes, and lastly, 2.3) to determine whether dietary LA supplementation can modulate age-dependent increases in iron levels in the brain.

**3.) To determine the effects of dietary lipoic acid-supplementation on the age-related changes in oxidative stress in cardiac myocytes.**

The age-related increase in oxidative stress is implicated in the decline in cardiac functions. However, most studies examining the effects of aging used isolated cardiac mitochondria which takes them out of the cellular context. Thus, in this study, we investigated the cellular consequences of mitochondrial decay on various oxidative parameters in isolated cardiac myocytes obtained from young and old rats.

**4.) To determine the extent of age-related changes in oxidative stress parameters in the two subpopulations of cardiac mitochondria.**

Cardiac mitochondria have distinct pattern of intracellular distribution, with one subpopulation lying beneath the sarcolemma (Subsarcolemmal mitochondria; SSM) and the other along the myofibrils (Interfibrillary mitochondria; IFM). In this aim, the patterns of age-related changes in mitochondrial functions were monitored by examining mitochondrial oxidant production, low molecular weight antioxidant status, indices of oxidative damage and resultant changes in mitochondrial electron transfer chain complex activity in IFM and SSM isolated from young and old rats (chapter 6).

**5.) To investigate the effects of lipoic acid administration on the age-related declines in hepatic glutathione homeostasis.**

Aging is associated with compromised antioxidant homeostasis. In particular, glutathione levels declines with age in many visceral organs, such as the brain and the liver. Intriguingly, our repeated experiments revealed that LA administrations lead to

reversal of the age-related declines in glutathione. Thus, in this study, we used LA as a tool to determine the mechanisms underlying age-related loss of glutathione homeostasis.

Overall, the results derived from this thesis show that dietary micronutrients have significant protective roles against transition metal ion and age-induced oxidative stress. Our results show that even under conditions that maximize its pro-oxidant potential, ascorbate strongly protected against iron and copper mediated lipid peroxidation. Moreover, we found novel effects of the dietary micronutrient, LA, in lowering chronically elevated levels of iron in the brain. Finally, our results show that treatment with lipoic acid (orally or via *i.p.* injection) improves cellular parameters of oxidative stress by acting as a potent activator of phase II enzymes. These studies provide novel *in vivo* modes of action for the dietary micronutrients in modulating oxidative stress.

**Table 1.1. Rate Constants for Scavenging Effects of DHLA Against Various ROS**

Reactive Species	Rate of scavenging	Reference
OH•	$1.92 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$	Matsugo et. Al., (1995) [50]
Singlet oxygen	$1.38 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$	Kaiser et Al., (1989)
Peroxyl radicals (AAPH)	$2.7 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$	Scott et al., (1994)[56]
Superoxide	$3.3 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$	Suzuki et al (1991)[59]

**Chapter 2.**

**Ascorbate does not act as a pro-oxidant towards lipids and proteins in human plasma exposed to excess transition metal ions and hydrogen peroxide**

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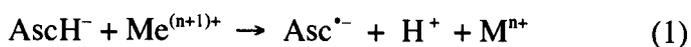
## 2.1 Abstract

The combination of ascorbate, transition metal ions and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is an efficient hydroxyl radical generating system called “the Udenfriend system”. Although the pro-oxidant role of ascorbate in this system has been well characterized in vitro, it is uncertain whether ascorbate also acts as a pro-oxidant under physiological conditions. To address this question, human plasma, used as a representative biological fluid, was either depleted of endogenous ascorbate with ascorbate oxidase, left untreated, or supplemented with 25  $\mu$ M–1 mM ascorbate. Subsequently, the plasma samples were incubated at 37°C with 50  $\mu$ M–1 mM iron (from ferrous ammonium sulfate), 60 or 100  $\mu$ M copper (from cuprous sulfate), and/or 200  $\mu$ M or 1 mM H<sub>2</sub>O<sub>2</sub>. – Although endogenous and added ascorbate was depleted rapidly in the presence of transition metal ions and H<sub>2</sub>O<sub>2</sub>, no cholesterol ester hydroperoxides or malondialdehyde were formed, i.e., ascorbate protected against, rather than promoted, lipid peroxidation. Conversely, depletion of endogenous ascorbate was sufficient to cause lipid peroxidation, the rate and extent of which were enhanced by the addition of metal ions but not H<sub>2</sub>O<sub>2</sub>. Ascorbate also did not enhance protein oxidation in plasma exposed to metal ions and H<sub>2</sub>O<sub>2</sub>, as assessed by protein carbonyl formation and depletion of reduced thiols. Interestingly, neither the rate nor the extent of endogenous  $\alpha$ -tocopherol oxidation in plasma was affected by any of the treatments. Our data show that even in the presence of redox-active iron or copper and H<sub>2</sub>O<sub>2</sub>, ascorbate acts as an antioxidant that prevents lipid peroxidation and does not promote protein oxidation in human plasma in vitro.

## 2.2 Introduction

Oxidative damage to biological macromolecules has been implicated in inflammation, aging and the pathogenesis of various chronic diseases, including atherosclerosis and certain types of cancer [9]. Low molecular weight antioxidants such as ascorbate (vitamin C),  $\alpha$ -tocopherol (vitamin E) and glutathione have been suggested to protect against these conditions by lowering the steady-state levels of oxidative damage to lipids, proteins and DNA [36,60].

Ascorbate is a potent water-soluble antioxidant capable of scavenging various types of reactive oxygen and nitrogen species [61]. Ascorbate has been shown to be the most effective water-soluble antioxidant in human plasma, preventing lipid peroxidation induced by, *e.g.*, aqueous peroxy radicals, activated neutrophils or the gas-phase of cigarette smoke [62-64]. However, in the presence of redox-active transition metal ions, ascorbate can also act as a pro-oxidant. *In vitro*, the combination of ascorbate ( $\text{AscH}^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and transition metal ions (Me), also referred to as “the Udenfriend system”, forms a highly pro-oxidant mixture generating hydroxyl radicals ( $\cdot\text{OH}$ ) that can oxidize almost any type of target molecule (RH) [65]:



Based on this pro-oxidant activity of ascorbate, concerns have been raised about supplemental vitamin C intake by individuals suffering from various conditions of iron

overload [66,67]. In contrast, recent animal and human studies have provided no evidence in support of a pro-oxidant activity of vitamin C *in vivo* [41,68-71].

In agreement with these observations, we have previously reported that the presence of redox-active, bleomycin-detectable iron and high concentrations of ascorbate in plasma of pre-term infants is not associated with increased steady-state concentrations of F<sub>2α</sub>-isoprostanes and protein carbonyls, biomarkers of *in vivo* lipid and protein oxidation, respectively [72]. In the same study, we also showed that ascorbate inhibited, rather than enhanced, lipid peroxidation in human plasma containing redox-active, bleomycin-detectable iron.

However, it could be argued that the lack of a pro-oxidant effect of ascorbate in our study [72] may have been due to the absence of H<sub>2</sub>O<sub>2</sub>, *i.e.*, the use of an incomplete Udenfriend system. Therefore, the purpose of the present study was to examine whether ascorbate acts as a pro- or anti-oxidant under the most oxidizing conditions where both redox-active iron and H<sub>2</sub>O<sub>2</sub> are present. Furthermore, we extended our previous observations to include measurement of protein carbonyls and reduced thiols, and use of the transition metal copper, which is known to be more potent than iron at catalyzing hydroxyl radical formation and lipid peroxidation [73]. Our results show that in the physiological environment of human plasma, ascorbate does not promote iron- or copper-induced lipid and protein oxidation but to the contrary acts as an antioxidant.

## 2.3 Materials and Methods

### *Preparation and incubation of human plasma*

Blood samples were collected into heparinized Vacutainer tubes from three healthy, normolipidemic subjects following an over-night fast. Plasma was obtained by centrifugation of blood at  $1000\times g$  for 20 min. All samples were spun twice to remove trace contamination of erythrocytes. For each set of experiments, plasma obtained from the same donor on three different occasions was used. Plasma samples were either left untreated, depleted of endogenous ascorbate by treatment with ascorbate oxidase (2.5 units/ml) for 5 min at room temperature, or supplemented with  $25\ \mu\text{M}$ – $1\ \text{mM}$  ascorbate. Ascorbate oxidase oxidizes ascorbate to dehydroascorbic acid without generation of  $\text{H}_2\text{O}_2$  [74]. Subsequently,  $50\ \mu\text{M}$ – $1\ \text{mM}$  iron (from ferrous ammonium sulfate), 60 or  $100\ \mu\text{M}$  copper (from cuprous sulfate) and/or  $200\ \mu\text{M}$  or  $1\ \text{mM}$   $\text{H}_2\text{O}_2$  was added, and the samples were incubated at  $37^\circ\text{C}$ . Although salts of the reduced metal ions were used, the metal ions undergo immediate autoxidation in solution under atmospheric oxygen [75,76]. Therefore, the metal ions added to plasma from stock solutions were in the oxidized form, i.e., ferric iron and cupric copper.

Aliquots were withdrawn from plasma incubations at specified time points and levels of cholesterol ester hydroperoxides (CEOOH), malondialdehyde (MDA), protein carbonyls, reduced thiols, ascorbate and  $\alpha$ -tocopherol were determined as described below. The amounts of iron added always exceeded the latent iron binding capacity of the plasma samples used ( $34.4 \pm 2.7\ \mu\text{M}$ ), resulting in 100% saturation of

transferrin and the availability of non-protein bound, redox-active iron [72]. Serum iron and latent iron binding capacity were measured with a commercial kit (Sigma procedure #565) based upon the determination of iron with ferrozine [77]. Total iron-binding capacity and transferrin saturation were calculated from those measured values [72].

#### *Ascorbate analysis*

Ascorbate was analyzed by paired-ion, reverse phase HPLC with electrochemical detection [62]. The plasma samples were acid-precipitated by mixing with an equal volume of 5% (w/v) metaphosphoric acid containing 1 mM of the metal chelator DTPA, and briefly centrifuged at 10,000×g. Forty  $\mu\text{l}$  of the supernatant was mixed with 12  $\mu\text{l}$  of 2.59 M  $\text{K}_2\text{HPO}_4$  buffer, pH 9.8, and 148  $\mu\text{l}$  of the HPLC mobile phase (40 mM sodium acetate, 0.54 mM DTPA, 1.5 mM dodecyltriethylammonium phosphate, 7.5% [v/v] methanol, pH 4.75). A 20- $\mu\text{l}$  aliquot of this mixture was injected into an LC-8 column (10 cm  $\times$  4.6 mm I.D.) (Supelco, Bellefonte, PA) preceded by a guard column (2 cm  $\times$  4.6 mm I.D.) containing the same packing material. The mobile phase was delivered at a flow rate of 1.0 ml/min. Ascorbate was detected at an applied potential of +600 mV using an LC 4C amperometric electrochemical detector equipped with a glassy carbon working electrode and a Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN). Before and after each set of analyses, a calibration was performed using a freshly prepared

standard solution of ascorbate in 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, containing 1 mM DTPA.

#### *Analysis of lipid hydroperoxides and $\alpha$ -tocopherol*

$\alpha$ -Tocopherol and CEOOH were detected in a single HPLC run with electrochemical and chemiluminescence detection, respectively [78]. Lipoprotein lipids and  $\alpha$ -tocopherol in plasma were extracted using a biphasic methanol/hexane mixture. One hundred  $\mu$ l of plasma was added to 500  $\mu$ l of methanol containing 1 mM DTPA and 5 ml of peroxide-free hexane (pre-treated with water). The sample was vortexed and briefly centrifuged at 800 $\times$ g. Four ml of the organic hexane phase was collected and dried under a constant stream of nitrogen at room temperature. The residue was reconstituted in 150  $\mu$ l mobile phase (ethanol/methanol/2-propanol [736:225:39, v/v/v] containing 0.8 g/l of LiClO<sub>4</sub>) and a 100- $\mu$ l aliquot was separated on an LC-18 column (25 cm  $\times$  4.6 mm I.D.) (Supelco). The mobile phase was delivered at a flow rate of 1.0 ml/min.  $\alpha$ -Tocopherol was detected at an applied potential of +500 mV, using a LC-4C amperometric electrochemical detector equipped with a glassy carbon working electrode and a Ag/AgCl reference electrode (Bioanalytical Systems). Following electrochemical detection, the eluate was mixed in a mixing-T with a post column mobile phase (100 mM sodium borate decahydrate, pH 10 : methanol [7:3, v/v], containing 174.4 mg/l of isoluminol and 5 mg/l of microperoxidase) prior to chemiluminescence detection in a Soma-3500 ChemiLumi Detector (Soma Optics Inc., Japan). The post column mobile phase was delivered at a

flow rate of 1.5 ml/min. Before and after each set of analyses, a calibration was performed using a standard mixture containing cholesterol linoleate hydroperoxide (made from purified cholesterol linoleate oxidized by 2,2'-azobis [2,4-dimethylvaleronitrile]) and  $\alpha$ -tocopherol from stock solutions stored at  $-80^{\circ}\text{C}$ .

#### *Analysis of malondialdehyde (MDA)*

Plasma MDA levels were measured by HPLC with fluorescence detection [79]. Briefly, 50  $\mu\text{l}$  plasma was mixed with 50  $\mu\text{l}$  of 0.2% butylated hydroxytoluene (BHT). Subsequently, 400  $\mu\text{l}$  of 0.44 M  $\text{H}_3\text{PO}_4$  acid and 100  $\mu\text{l}$  of 44 mM 2-thiobarbituric acid containing 0.1 M sodium sulfate was added, and the samples were incubated at  $90^{\circ}\text{C}$  for 45 min. Following incubation, the samples were cooled on ice for 5 min, and 250  $\mu\text{l}$  n-butanol was added. The samples were centrifuged at  $16,000\times g$  for 5 min and the n-butanol phase was collected. Twenty-five  $\mu\text{l}$  of the extract was separated on a spherisorb ODS-2 column (Supelco) using a mobile phase consisting of methanol and potassium phosphate buffer (pH 6.8, 6:4, v/v). The fluorimeter was set at an excitation wavelength of 512 nm and an emission wavelength of 532 nm. The TBA-MDA content was authenticated and quantified using 1,1,3,2-tetraethoxypropane as the external standard.

#### *Analysis of protein carbonyls*

Protein carbonyls were assayed as described by Levine et al. [80]. Briefly, 50  $\mu\text{l}$  plasma was mixed with 500  $\mu\text{l}$  of 10 mM 2,4,-dinitrophenylhydrazine (DNPH) in 2

N HCl. The mixture was incubated at room temperature for 1 h, followed by the addition of 0.5 ml 20% trichloroacetic acid. The samples were incubated on ice for 10 min and centrifuged at 3000×g for 10 min. The protein pellets were washed three times with 3 ml ethanol : ethyl acetate (1:1, v/v) and dissolved in 1 ml 6 M guanidine (pH 2.3). The peak absorbance at 370 nm was measured to quantify protein carbonyls. The data are expressed as nmol of carbonyls/mg protein, using a molar absorption coefficient of 22,000 M<sup>-1</sup>cm<sup>-1</sup> for the DNPH derivatives.

#### *Analysis of reduced thiols*

Reduced thiol content in plasma was determined by a spectrophotometric assay using 2,2-dithiobisnitrobenzoic acid. Briefly, 50 μl plasma was mixed with 1 ml of Tris-EDTA buffer (0.25 M Tris base-20 mM EDTA; pH 8.2). Subsequently, 20 μl of a DTNB solution (4 mg/ml in absolute methanol) was added and the samples were incubated for 15 min. The absorbance of the sample at 412 nm was measured and subtracted from a DTNB blank and a blank containing the sample without DTNB. The molar absorption coefficient of 13,600 M<sup>-1</sup>cm<sup>-1</sup> was used for quantification.

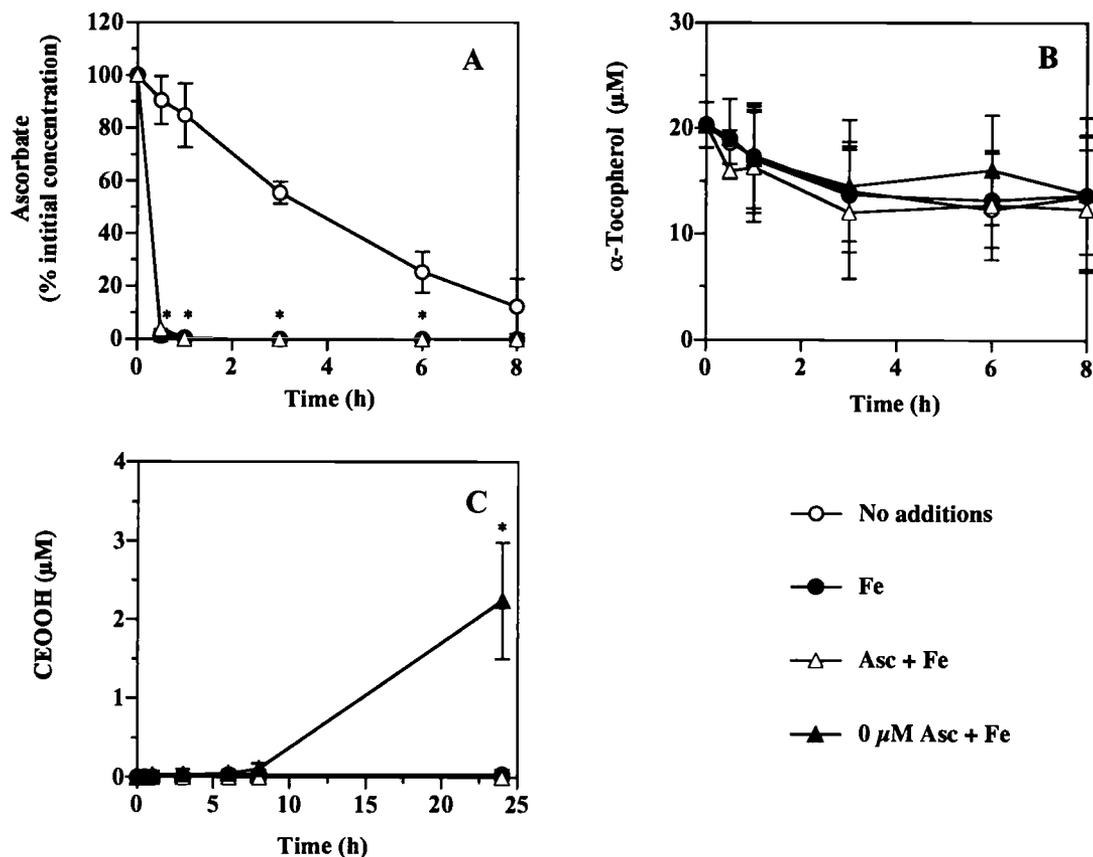
#### *Statistical Analysis*

Statistical significance was determined by single factor ANOVA with Tukey's post-hoc test, using Statview statistical software. Results are expressed as the mean ± SEM. A p-value of less than 0.05 was considered significant.

## 2.4 Results

To investigate the role of ascorbate in metal ion-dependent lipid peroxidation under physiological conditions, ascorbate concentrations in human plasma were manipulated and iron-induced changes in CEOOH, ascorbate and  $\alpha$ -tocopherol were determined. In control plasma containing  $48.7 \pm 11.0 \mu\text{M}$  endogenous ascorbate and no added iron, ascorbate was consumed slowly ( $3.1 \pm 1.1 \mu\text{M}/\text{min}$  [mean  $\pm$  S.D.,  $n = 3$ ];  $t_{1/2} = 3.5 \text{ h}$ ) (Fig. 2.1A). In contrast, addition of  $50 \mu\text{M}$  iron caused rapid and complete oxidation of endogenous and added ascorbate ( $25, 50$  or  $75 \mu\text{M}$ ) within less than  $1 \text{ h}$  (Fig. 1A). The initial rate of oxidation of endogenous  $\alpha$ -tocopherol was much lower ( $0.07 \pm 0.09 \mu\text{M}/\text{min}$  [mean  $\pm$  S.D.,  $n = 3$ ];  $t_{1/2} \geq 8 \text{ h}$ ) than that of ascorbate and was not affected by changes in iron or ascorbate status (Fig. 2.1B).

Although the added iron interacted with ascorbate, causing its rapid oxidation (Fig. 1A), no CEOOH could be detected for up to  $24 \text{ h}$  of incubation (Fig. 2.1C). Furthermore, MDA levels in plasma incubated for  $24 \text{ h}$  with  $50 \mu\text{M}$  iron ( $0.48 \pm 0.05 \mu\text{M}$ ) were not different from MDA levels in control plasma incubated without added iron ( $0.45 \pm 0.02 \mu\text{M}$ ). These results suggest that the absence of detectable CEOOH in plasma treated with iron (Fig. 2.1C) is not due to iron-mediated breakdown of lipid hydroperoxides. Iron-dependent lipid peroxidation could only be detected in plasma that had been depleted of endogenous ascorbate, and only after prolonged incubation (Fig. 2.1C). Consistent with this observation, iron-mediated increases in plasma MDA



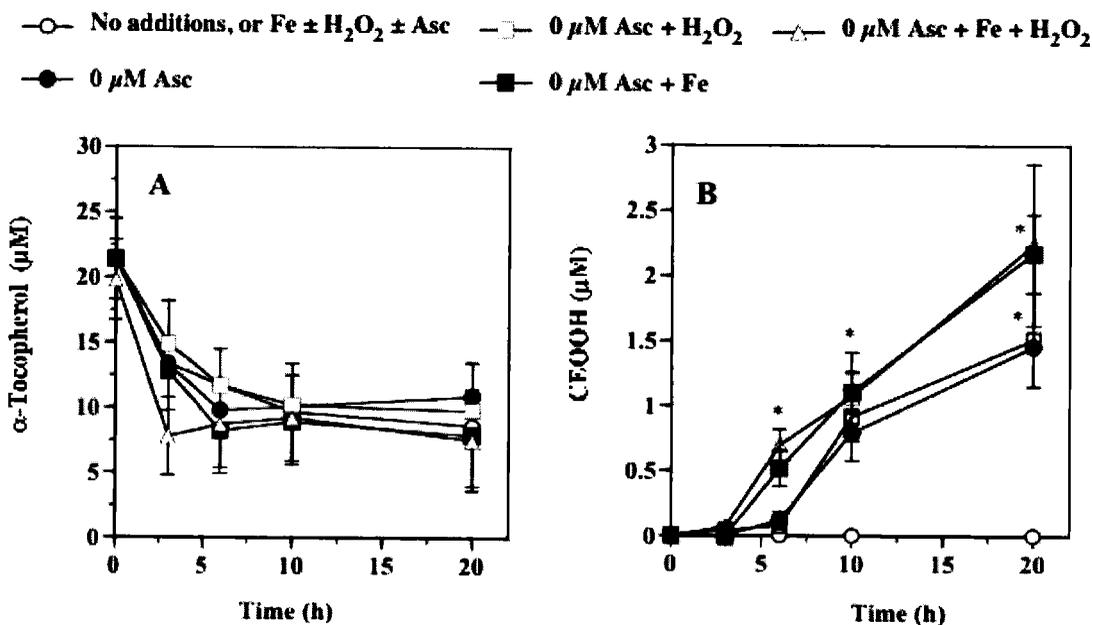
**Figure 2.1. Ascorbate prevents lipid peroxidation in human plasma exposed to excess iron.**

Freshly isolated plasma ( $48.7 \pm 11.0 \mu\text{M}$  endogenous ascorbate) was either left untreated, depleted of endogenous ascorbate (“0  $\mu\text{M}$  Asc”), or supplemented with 75  $\mu\text{M}$  ascorbate, resulting in measured ascorbate concentrations of 48.7, <1, and 124.8  $\mu\text{M}$ , respectively. Subsequently, 50  $\mu\text{M}$  iron (Fe) was added and the samples were incubated at 37°C. Ascorbate (panel A),  $\alpha$ -tocopherol (panel B) and cholesterol ester hydroperoxides (CEOOH; panel C) were measured as described in Methods. Similar results were obtained when 25 or 50  $\mu\text{M}$  ascorbate was added (data not shown). Values are the mean  $\pm$  S.E.M. from three experiments, using plasma obtained from the same subject (donor #1) on three different occasions. \*Denotes values that are significantly different ( $p < 0.05$ ) from the “no additions” control.

levels were only observed in plasma depleted of endogenous ascorbate ( $0.74 \pm 0.07 \mu\text{M}$ ). These results confirm that even in the presence of redox-active iron, ascorbate protects against iron-induced lipid peroxidation in human plasma [72].

*In vitro*, ascorbate may enhance oxidative damage by reducing ferric to ferrous ions, which in turn can reduce  $\text{H}_2\text{O}_2$  to hydroxyl radicals. Therefore, we examined the role of ascorbate in lipid peroxidation in the presence of both redox-active iron and  $\text{H}_2\text{O}_2$  (the Udenfriend system). The concentrations of iron and ascorbate used in these experiments were increased from those used above (Fig. 2.1) to maximize the probability of detecting a pro-oxidant effect of ascorbate. Addition of  $100 \mu\text{M}$  iron and/or  $200 \mu\text{M}$   $\text{H}_2\text{O}_2$  to plasma caused rapid and complete oxidation of endogenous ( $83.2 \pm 6.7 \mu\text{M}$ ) and added ascorbate ( $200 \mu\text{M}$ ) within less than 3 h of incubation (data not shown). Similar to the above experiments (Fig. 2.1B), the rate of oxidation of  $\alpha$ -tocopherol was much lower than that of ascorbate and was not significantly affected by the addition of iron,  $\text{H}_2\text{O}_2$  or ascorbate (Fig. 2.2A).

If ascorbate was initially present, CEOOH formation could not be detected throughout the 20-h incubation (Fig. 2.2B). Even under the most “oxidizing” conditions, *i.e.*, iron,  $\text{H}_2\text{O}_2$  and ascorbate added together, no CEOOH could be detected (Fig. 2.2B) and MDA remained at control levels ( $0.32 \pm 0.06 \mu\text{M}$ ). In contrast, depletion of endogenous ascorbate was sufficient to cause lipid peroxidation, as indicated by significant increases in plasma CEOOH (Fig. 2.2B) and MDA ( $1.29 \pm 0.08 \mu\text{M}$ ). Furthermore, adding iron and  $\text{H}_2\text{O}_2$  to plasma devoid of ascorbate resulted in higher MDA levels ( $0.85 \pm 0.04 \mu\text{M}$ ) than those observed in plasma containing



**Figure 2.2. Ascorbate prevents lipid peroxidation in human plasma exposed to excess iron and hydrogen peroxide.**

Freshly isolated plasma ( $83.2 \pm 6.7 \mu\text{M}$  endogenous ascorbate) was either left untreated, depleted of endogenous ascorbate (“0  $\mu\text{M}$  Asc”), or supplemented with 200  $\mu\text{M}$  ascorbate. Subsequently, 200  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> and/or 100  $\mu\text{M}$  iron (Fe) was added and the samples were incubated at 37°C.  $\alpha$ -Tocopherol (panel A) and cholesterol ester hydroperoxides (CEOOH; panel B) were measured as described in Methods. Values are the mean  $\pm$  S.E.M. from three experiments, using plasma obtained from the same subject (donor #2) on three different occasions. \*Denotes values that are significantly different ( $p < 0.05$ ) from the “no additions” control.

endogenous ascorbate ( $0.55 \pm 0.02 \mu\text{M}$ ). The lack of a significant increase in plasma MDA in samples treated with iron and ascorbate suggests that the absence of CEOOH in these samples is not due to enhanced breakdown of lipid hydroperoxides. Taken together, the findings show that even in the presence of the Udenfriend system, ascorbate protects against, rather than enhances, iron-dependent lipid peroxidation in human plasma.

To confirm that ascorbate acts as an antioxidant towards lipids in plasma exposed to redox-active transition metal ions, copper was used instead of iron. As expected, addition of  $60 \mu\text{M}$  copper resulted in complete oxidation of both endogenous ( $83.2 \pm 6.7 \mu\text{M}$ ) and added ascorbate ( $200 \mu\text{M}$ ) within 3 h of incubation (data not shown) but did not affect  $\alpha$ -tocopherol oxidation (Fig. 2.3A). Despite the rapid oxidation of ascorbate, CEOOH were not detected throughout the 20-h incubation, even when copper,  $\text{H}_2\text{O}_2$  and ascorbate were added together (Fig. 2.3B). Consistent with the above data (Figs. 2.1C and 2.2B), lipid peroxidation only occurred in plasma that had been depleted of endogenous ascorbate, and was somewhat enhanced by the addition of copper but not  $\text{H}_2\text{O}_2$  (Fig. 2.3B). Furthermore, ascorbate decreased, rather than increased, plasma MDA formation induced by copper and  $\text{H}_2\text{O}_2$  ( $0.56 \pm 0.02$  vs.  $0.78 \pm 0.05 \mu\text{M}$  in the presence and absence of endogenous ascorbate, respectively). These data establish that ascorbate protects against transition metal ion-dependent lipid peroxidation in plasma, even in the presence of  $\text{H}_2\text{O}_2$ .

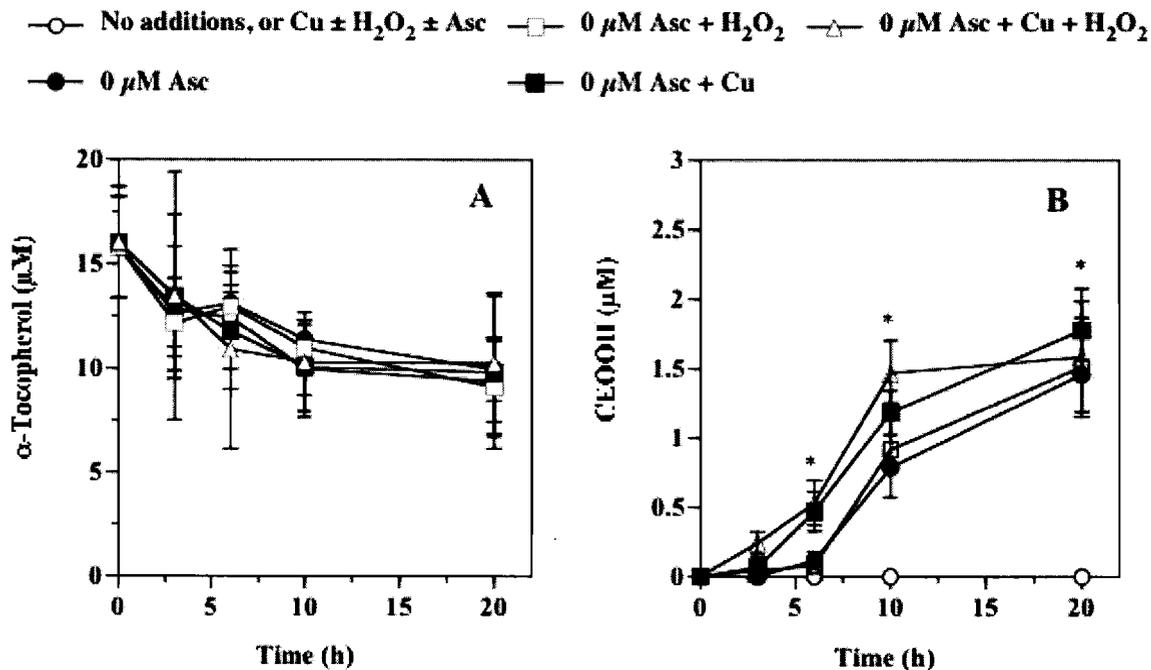
Although the above results demonstrate an antioxidant role of ascorbate against metal ion-dependent lipid peroxidation, it is possible that ascorbate enhances

oxidation of other target molecules in plasma. Metal ions are known to bind to proteins and can cause site-specific oxidative damage, which may be enhanced by ascorbate [81]. To address this possibility, protein oxidation was measured by protein carbonyl formation. Control plasma samples incubated for 96 h at 37°C contained about 200  $\mu\text{M}$  protein carbonyls (see Fig. 2.4). However, these levels were not increased by addition of 100  $\mu\text{M}$  iron, 100  $\mu\text{M}$  copper, or 1 mM  $\text{H}_2\text{O}_2$  (data not shown). Even in the presence of the Udenfriend system, containing either iron or copper, protein carbonyl formation was not increased (data not shown).

As the above results remained inconclusive regarding a potential pro-oxidant effect of ascorbate towards plasma proteins, even higher concentrations of the reactants were used, *viz.*, 1 mM ascorbate and 0.25, 0.5 and 1 mM iron, or plasma was depleted of endogenous ascorbate with ascorbate oxidase. In plasma devoid of ascorbate, addition of iron caused a dose-dependent increase in protein carbonyls (Fig. 4). However, iron-mediated protein oxidative damage was neither enhanced nor reduced by the addition of ascorbate (Fig. 2.4). Addition of the Udenfriend system also did not enhance protein carbonyl formation above the level seen in the presence of iron alone, or iron and ascorbate (Fig. 2.4). These data suggest that ascorbate, while unable to protect against iron-dependent protein oxidation in plasma, does not enhance it, even in the presence of redox-active metal ions and  $\text{H}_2\text{O}_2$ .

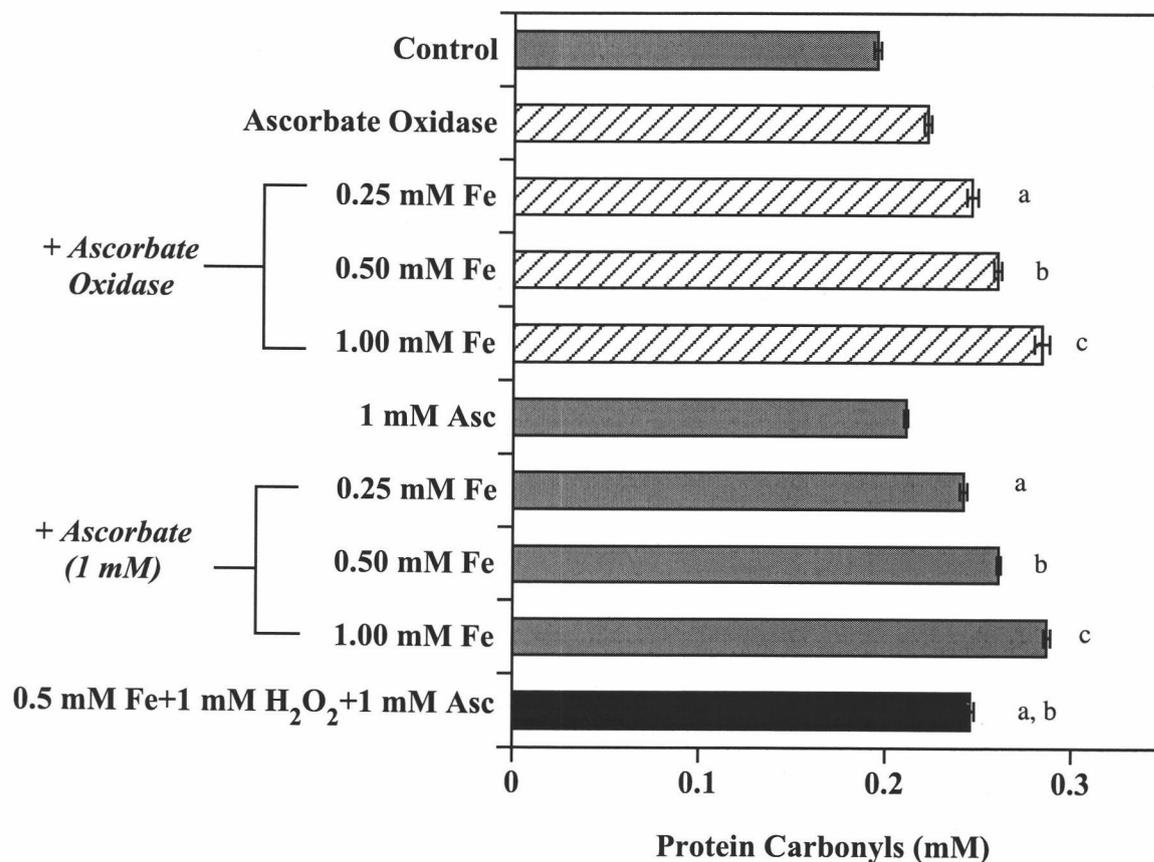
To further examine the role of ascorbate in modulating metal ion-dependent protein oxidation, changes in plasma reduced thiols were determined. Human albumin contains 35 cysteine residues, 34 of which form intramolecular disulfide bridges. The

only reduced cysteine residue in albumin makes up the bulk of reduced thiols in plasma [62]. The reduced thiol content in control plasma was 336  $\mu\text{M}$ , which was decreased to 252  $\mu\text{M}$  by treatment with 1mM  $\text{H}_2\text{O}_2$ . Incubation of plasma with the Udenfriend system (100  $\mu\text{M}$  iron, 200  $\mu\text{M}$  ascorbate, 1 mM  $\text{H}_2\text{O}_2$ ) resulted in less thiol loss than incubation with  $\text{H}_2\text{O}_2$  alone, with reduced thiol levels (321  $\mu\text{M}$ ) remaining close to those observed in control plasma. Thus, like protein carbonyl formation (Fig. 2.4), plasma thiol oxidation was not enhanced by ascorbate in the presence of  $\text{H}_2\text{O}_2$  and iron, confirming that ascorbate does not act as a pro-oxidant under these conditions.



**Figure 2.3. Ascorbate prevents lipid peroxidation in human plasma exposed to copper and hydrogen peroxide.**

Freshly isolated plasma ( $83.2 \pm 6.7 \mu\text{M}$  endogenous ascorbate) was either left untreated, depleted of endogenous ascorbate ("0  $\mu\text{M}$  Asc"), or supplemented with 200  $\mu\text{M}$  ascorbate. Subsequently, 200  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> and/or 60  $\mu\text{M}$  copper (Cu) was added and the samples were incubated at 37°C.  $\alpha$ -Tocopherol (panel A) and cholesterol ester hydroperoxides (CEOOH; panel B) were measured as described in Methods. Values are the mean  $\pm$  S.E.M. from three experiments, using plasma obtained from the same subject (donor #2) on three different occasions. \*Denotes values that are significantly different ( $p < 0.05$ ) from the "no additions" control.



**Figure 2.4.** Ascorbate does not enhance protein oxidation in human plasma exposed to iron and hydrogen peroxide.

Freshly isolated plasma was either left untreated, depleted of endogenous ascorbate ( $73.0 \pm 8.5 \mu\text{M}$  endogenous ascorbate) with ascorbate oxidase, or supplemented with 1 mM ascorbate. Subsequently, 1 mM  $\text{H}_2\text{O}_2$  and/or 0.25, 0.5 or 1 mM iron (Fe) were added and the samples were incubated at  $37^\circ\text{C}$  for 96 h. Protein carbonyls were measured as described in Methods. Values are the mean  $\pm$  S.E.M. from three experiments, using plasma obtained from the same subject (donor #3) on three different occasions. Values are significantly different ( $p < 0.05$ ) from each other unless they share the same letter.

## 2.5 Discussion

Due to the potentially harmful interactions between ascorbate and iron, which may cause oxidative damage to biological macromolecules, concerns have been raised about supplemental vitamin C intake by individuals with high iron status or clinical iron overload [67,82]. In the present study, we examined whether ascorbate acts as a pro- or anti-oxidant in the presence of redox-active transition metal ions and  $H_2O_2$ , using freshly isolated human plasma as a physiological fluid. The effects of different ascorbate concentrations on transition metal ion-dependent oxidation of plasma antioxidants, lipids and proteins in the presence or absence of  $H_2O_2$  were determined. Our results show that vitamin C does not act as a pro-oxidant towards lipids and proteins in human plasma *in vitro*, even under the most “oxidizing” conditions of the Udenfriend system (metal ion, ascorbate,  $H_2O_2$ ).

The notion that ascorbate acts as a pro-oxidant in the presence of transition metal ions is based on the ability of ascorbate to enhance metal ion-dependent hydroxyl and alkoxy radical formation *in vitro* by Fenton chemistry [see reactions (1) and (2)]. However, due to the presence of various metal binding proteins such as transferrin, ferritin and ceruloplasmin, the availability of “free”, redox-active metal ions in plasma and other biological fluids is very low [8]. For example, Minetti *et al.* [83] found that hydroxyl radical formation from 50–300  $\mu M$   $H_2O_2$  in iron-overloaded human plasma could only be detected if EDTA was added to mobilize iron from physiological ligands, such as proteins and citrate. Furthermore, several animal studies

have shown that iron-overload is not associated with increased lipid peroxidation *in vivo* [68,69,84]. The same inability of transition metal ions to enhance lipid peroxidation was also observed in apo E knockout mice [70], a strain of mice prone to atherosclerosis. These data suggest that transition metal ions do not contribute significantly to increased oxidative stress *in vivo*.

The antioxidant activity of ascorbate against metal ion-dependent lipid peroxidation observed in the present study may be due to two main factors. First, ascorbate may directly scavenge free radicals and prevent them from initiating lipid peroxidation. Previous studies in human plasma have shown that ascorbate forms the first line of antioxidant defense against various aqueous radicals and oxidants [38,63,85]. Our results also show rapid ascorbate oxidation occurring immediately following the addition of metal ions, which is likely due metal ion-mediated ascorbate “autoxidation” and free radical scavenging. Ascorbate can both chelate and reduce transition metal ions, and the reduced metal ion in turn can reduce oxygen or H<sub>2</sub>O<sub>2</sub> to superoxide and hydroxyl radicals, respectively [see reactions (1) and (2)]. Superoxide and hydroxyl radicals are scavenged by ascorbate with second order rate constants of  $1 \times 10^5$  and  $1.1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ , respectively [61].

Second, ascorbate may inhibit metal ion binding to LDL and other lipoproteins in plasma subsequent to site-specific destruction of metal-binding sites on these lipoproteins. Our previous studies have shown that ascorbate prevents copper-dependent LDL oxidation by destruction of specific metal binding sites on apo B, in particular histidine residues [86,87]. Once the metal ion is released from the

lipoprotein, it can no longer oxidize its lipids [87] but instead may bind to other proteins in plasma or generate hydroxyl radicals from  $H_2O_2$  in solution. As indicated above, these hydroxyl radicals may be effectively scavenged by ascorbate as well as other plasma antioxidants, such as urate, bilirubin and glucose [63].

*In vitro*, ascorbate can recycle  $\alpha$ -tocopherol from the  $\alpha$ -tocopheroxyl radical [88], however, the *in vivo* relevance of these observations is unclear. The experimental evidence from a number of human and animal supplementation studies does not support a synergistic interaction between ascorbate and  $\alpha$ -tocopherol [89,90]. In the present study, we observed that varying the ascorbate concentration, and even depleting ascorbate, did not change the rate of  $\alpha$ -tocopherol oxidation in human plasma. Conversely, other investigators have shown that varying the plasma  $\alpha$ -tocopherol concentration does not alter the rate of ascorbate oxidation [91]. Furthermore, we found that the rate of  $\alpha$ -tocopherol oxidation was not enhanced by the addition of metal ions or  $H_2O_2$ , and lipid peroxidation was contingent upon the absence of ascorbate but not  $\alpha$ -tocopherol. These data show that ascorbate prevents lipid peroxidation independently of  $\alpha$ -tocopherol, and  $\alpha$ -tocopherol is ineffective at preventing metal ion-induced lipid peroxidation in plasma *in vitro*.

In contrast to studies on other oxidative biomarkers, there are only few studies that have examined the role of ascorbate in metal ion-mediated protein oxidation. Although some investigators have found that ascorbate can enhance protein glycooxidation [92], others have reported that ascorbate can lower copper-dependent dityrosine formation [93]. Studies using human plasma generally have found that

ascorbate does not protect against protein thiol oxidation or carbonyl formation induced by a number of non-metal ion oxidative stressors, such as cigarette smoke, aqueous radicals generated by 2,2'-azobis(2-amidinopropane) hydrochloride, or hypochlorous acid [39,63,94]. Human supplementation studies also do not show a strong correlation between plasma levels of ascorbate and protein oxidation markers [93,95]. Consistent with these observations, we found that modulating ascorbate concentrations did not result in significant changes in the levels of protein carbonyls or reduced thiols in human plasma exposed to metal ions and H<sub>2</sub>O<sub>2</sub>.

In conclusion, our data show that the pro-oxidant activity of ascorbate in the Udenfriend system does not apply to the physiological environment of plasma. Thus, ascorbate in human plasma does not enhance protein and lipid oxidation, even in the presence of redox-active iron or copper and H<sub>2</sub>O<sub>2</sub>. To the contrary, our current and previous data [72] show that ascorbate strongly protects against metal ion-mediated lipid peroxidation in plasma *in vitro*, whereas  $\alpha$ -tocopherol is ineffective.

## **2.6 Acknowledgement**

This work was supported by grants HL-60886 and AT-00066 from the National Institutes of Health, and pre-doctoral fellowship 9910089Z from the American Heart Association.

### **Chapter 3.**

**Dihydrolipoic acid lowers the redox activity of transition metal ions but does not  
remove them from enzymes**

Jung Suh, Evan deSzoeker, Benzhan Zhu, Balz Frei and Tory M. Hagen

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### 3.1 Abstract

$\alpha$ -Lipoic acid (LA) and its reduced form, dihydrolipoic acid (DHLA), have been suggested to chelate transition metal ions, and hence, may be beneficial in lowering iron or copper-mediated oxidative stress in biological systems. However, it remains unclear whether LA and DHLA chelate transition metal ions in a redox-inactive form and whether they can also remove metal ions from active sites of enzymes. To address these questions, we first investigated the effects of LA and DHLA on iron or copper-catalyzed oxidation of ascorbate. We found that DHLA slightly inhibited Fe(III)-citrate mediated ascorbate oxidation in a concentration-dependent manner. In contrast, no significant inhibition was observed with LA, suggesting that reduced thiols are required for binding of Fe(III). When Cu(II)(histidine)<sub>2</sub> was used, DHLA strongly inhibited ascorbate oxidation in a concentration-dependent manner, with complete inhibition at a DHLA:Cu(II) ratio of 3. In contrast, no inhibition was observed with LA. To study whether LA or DHLA remove copper or iron ions from proteins, the changes in CuZn superoxide dismutase and aconitase activities in the presence or absence of increasing concentrations of LA or DHLA were examined. We found that neither LA nor DHLA altered CuZn-SOD or aconitase activity. Thus, DHLA, but not LA, may be beneficial in chelating and inactivating redox-active transition metal ions, in particular copper, without removing protein-bound iron and copper, and thus leaving metal-dependent enzyme activities intact.

### 3.2 Introduction

Disruptions in iron and copper homeostasis and resultant oxidative stress have been implicated in the pathogenesis of a number of diseases [96-98]. The potential toxic effects of iron and copper suggest that chelation to remove excess metals *in vivo* may be beneficial, *e.g.*, in the treatment of Alzheimer's, Parkinson's and Wilson's disease. However, extensive use of currently available chelating agents such as EDTA, desferrioxamine (DFO), penicillamine, and dimercaptosuccinic acid is sometimes hampered by inefficacy, cost and toxic side effects associated with their use [99,100].

$\alpha$ -Lipoic acid (LA) is a cofactor for  $\alpha$ -keto acid dehydrogenases that are essential for carbohydrate and lipid catabolism. *In vivo*, exogenously supplied LA is rapidly reduced to dihydrolipoic acid (DHLA) and both can readily cross the blood brain barrier [101]. The low reduction potential (-0.32 V) of the LA/DHLA redox couple makes DHLA capable of regenerating other low molecular weight antioxidants such as ubiquinol (coenzyme Q) [44] and ascorbate (vitamin C) [49]. Aside from these antioxidant properties, LA and DHLA have also been claimed to exhibit metal chelating properties [53].

The potential use of LA as a therapeutic metal-chelating agent has been previously proposed [54,56,102]. In a polar but non-aqueous solution, LA and DHLA bind a number of different metals, including iron and copper [53]. Structurally, the two thiol groups and the carboxyl group of LA allow binding of divalent metals

[53,58]. Such chelation by LA appears to be effective in slowing iron or copper-mediated oxidation of lipids in various model systems [54,55,103].

However, some studies have found pro-oxidant effects of DHLA in the presence of transition metal ions. For example, *in vitro* DHLA mobilizes iron from ferritin [58], and may cause an increase in intracellular free iron. Also, DHLA may act as a pro-oxidant by reducing Fe(III) to Fe(II) and enhancing liposome oxidation [57] and deoxyribose degradation [56].

The potential pro-oxidant effects of DHLA raise the question of whether LA and DHLA chelate transition metal ions in a redox-inactive form. In addition, it remains to be studied whether LA or DHLA can remove enzyme-bound metals. Thus, the aims of the current study were 1) to examine and compare the capacity of LA and DHLA to modulate the redox-activities of iron and copper complexes, and 2) to investigate whether LA and DHLA can remove copper and iron from proteins such as Cu,Zn- SOD and aconitase, thereby affecting enzyme activity.

### 3.3 Materials and Methods

#### *Materials*

All chemical reagents and enzymes used were purchased from Sigma (St. Louis, MO). The PD10 columns and Chelex-100 resins were purchased from Bio-Rad Laboratories (Hercules, CA).

#### *Iron and Copper-catalyzed Ascorbate Oxidation*

Ascorbate (100  $\mu\text{M}$ ) was incubated in 10 mM chelex-treated potassium phosphate buffer (pH 7.4) at 37°C in the absence or in the presence of either LA (1-100  $\mu\text{M}$ ) or DHLA (1-50  $\mu\text{M}$ ). To these samples, either 50  $\mu\text{M}$  Fe(III)-citrate or 1  $\mu\text{M}$  Cu(II)(histidine)<sub>2</sub> were added to initiate ascorbate oxidation. The rate of ascorbate loss was monitored over 10 -15 min by following the absorbance loss at 265 nm in a Beckman DU-640 spectrophotometer.

#### *Superoxide Dismutase Activity*

The ability of LA and DHLA to remove copper ions from CuZn SOD was determined by monitoring changes in enzyme activity. Purified bovine liver SOD (2  $\mu\text{g}/\text{ml}$ ) was incubated at 37°C in 0.05 M Tris-Cl (pH 8.2) containing 0.05 M DTPA, with or without 0.1 or 0.5 mM DHLA, LA, diethyldithiocarbamate (DDC), or tetraethylthiuram disulfide (TTD). The reaction was initiated by adding 25  $\mu\text{l}$  of a

freshly prepared stock solution of pyrogallol (1,2,3-trihydroxybenzene; 12 mM in 10 mM HCl) to 1 ml of the incubation mixture (final pyrogallol concentration, 300  $\mu$ M). The rate of pyrogallol autoxidation was measured by monitoring the absorbance change at 420 nm [104]. One unit of SOD activity was defined as the amount of SOD needed for 50% inhibition of pyrogallol oxidation.

#### *Aconitase Activity*

Aconitase activity was determined according to Fujita *et al* [105]. To minimize the presence of apo-aconitase, purified porcine heart aconitase (2.5 mg/ml) was incubated in activation buffer (50 mM Tris buffer containing 5 mM DTT, 20  $\mu$ M ferrous ammonium sulfate, and 12.5  $\mu$ M Na<sub>2</sub>S<sub>2</sub>) at 37°C for 30 minutes. Excess iron and reducing agents were removed using PD10 columns, and protein levels were quantified with the Lowry assay. The activated aconitase (0.25 mg/ml) was incubated for 30 min without or with either LA (0.2 – 2.0 mM) or DHLA (0.5 – 5 mM). Aconitase activity was determined by adding an aliquot of the incubation mixture (200  $\mu$ l) to 1.0 ml of assay buffer (50 mM Tris buffer containing 0.2 mM citrate, 0.5 mM NADP, 0.05 mM MgSO<sub>3</sub> and 0.2 mg of isocitric dehydrogenase). The rate of NADPH formation was monitored at 340 nm for 10 minutes. Under these conditions, maximal activity of aconitase ( $18 \pm 4$   $\mu$ moles/min/mg protein) was maintained for at least 90 min.

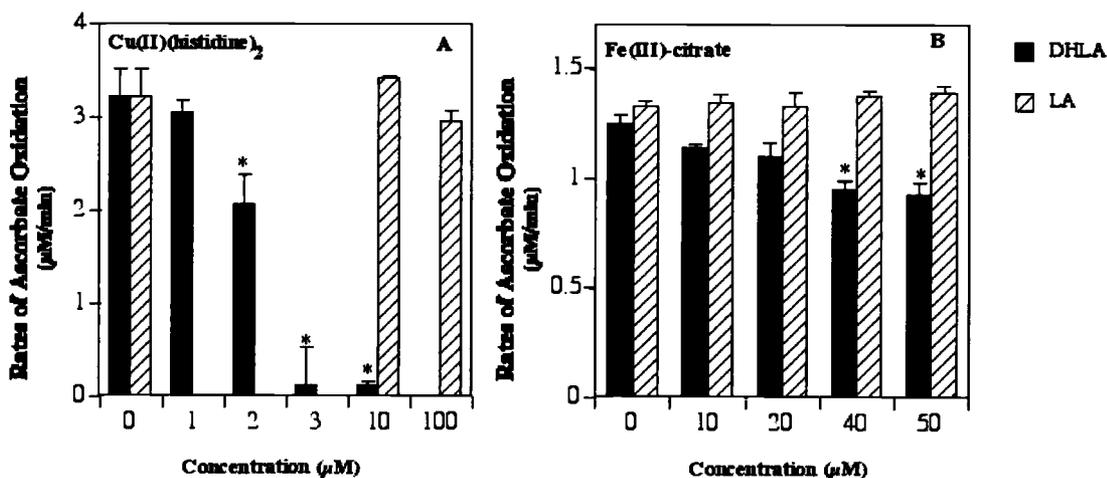
### *Statistical Analysis*

Statistical significance was determined by single factor ANOVA with Tukey's post-hoc test, using statview statistical software. Results are expressed as the mean  $\pm$  SEM. A P-value of less than 0.05 was considered significant.

### 3.4 Results

The ability of LA and DHLA to chelate copper and iron ions in a redox-inactive form was assessed by investigating their effects on copper or iron-catalyzed ascorbate oxidation. As shown in Figure 3.1A, addition of Cu(II)(histidine)<sub>2</sub> (1 μM) to ascorbate (100 μM) caused rapid oxidation of ascorbate (-3.21±0.3 μM/min). DHLA inhibited ascorbate oxidation in a concentration-dependent manner and rendered complete inhibition at a DHLA:Cu(II) molar ratio of 3 (-0.11±0.4 μM/min; p<0.0001; Figure 3.1A). Despite strong protection by DHLA, a similar inhibition of Cu(II)-mediated ascorbate oxidation was not observed with LA, even when the concentration of LA was 100-fold greater than Cu(II) (-3.21±0.3 μM/min versus -3.42±0.02 μM/min in the absence or presence of 100 μM LA, respectively; Figure 3.1B). These results show that DHLA, but not LA, can chelate copper in a redox inactive manner.

Figure 3.1B shows the effects of LA and DHLA on iron-mediated ascorbate oxidation. As expected, Fe(III)-citrate (50 μM) oxidized ascorbate (100 μM) at a much slower rate compared to copper (-1.25±0.06 μM/min versus -3.21±0.3 μM/min in the presence of Fe(III) or Cu(II), respectively; Figure 3.1B). As in case of copper, adding increasing concentrations of DHLA inhibited iron-mediated ascorbate oxidation in a concentration dependent manner (Figure 3.1B). At a DHLA:iron molar ratio of 1:1, a significant 26% (P<0.002) inhibition of ascorbate oxidation (Figure 3.1B). Similar to results in Figure 3.1B, LA did not have any effect towards iron-



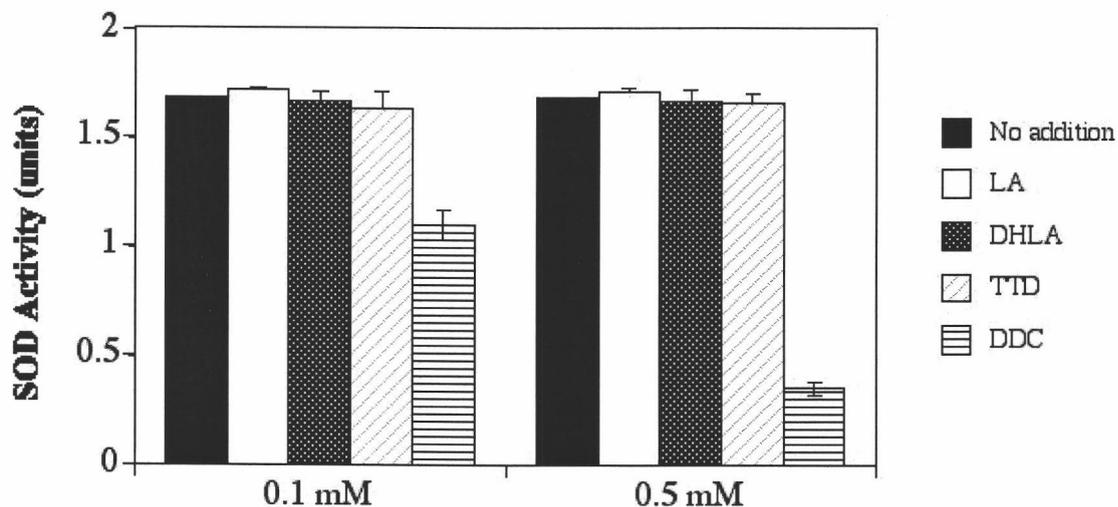
**Figure 3.1. The effect of DHLA and LA on the redox activities of Cu(II)(Histidine)<sub>2</sub> and Fe(III)-citrate.**

The effect of DHLA and LA on the redox activities of Cu(II)(Histidine)<sub>2</sub> and Fe(III)-citrate were determined by monitoring ascorbate oxidation. In panel (A), 100 μM ascorbate was incubated with or without 1, 2, 3, or 10 μM DHLA or 10 or 100 μM LA. To these samples, 1 μM Cu(II)(Histidine)<sub>2</sub> was added and the rate of ascorbate loss was determined by following the absorbance at 265 nm for 15 min. Results show a complete inhibition of Cu(II)-mediated ascorbate oxidation when the molar ratio of DHLA to Cu(II) is greater or equal to three but LA was ineffective. In panel (B), experiments in panel A were repeated with Fe(III)citrate replacing Cu(II)(Histidine)<sub>2</sub>. 10,20, 40, and 50 μM LA or DHLA were used. Results show a small but significant inhibition of Fe(III)-dependent ascorbate oxidation when the molar ratio between DHLA and Fe(III) is greater than or equal to 0.75. LA was ineffective in modulating the redox activities of Fe(III)citrate. Results are expressed as mean ± SD. (\* denotes statistically significant differences from controls)

mediated ascorbate oxidation (Figure 3.2B). These results show that DHLA, but not LA slightly lowers redox activity of iron ions.

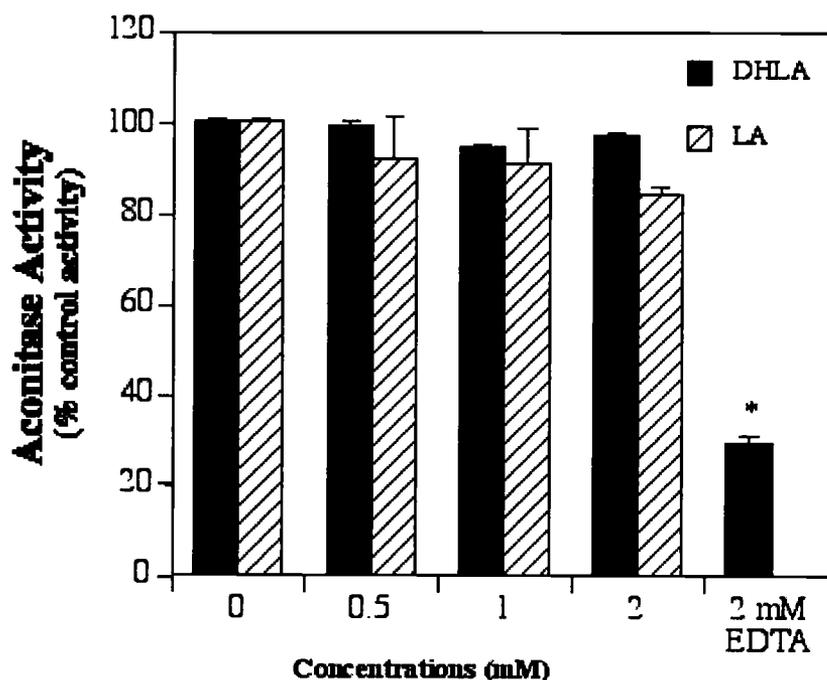
The above experiments show that DHLA strongly binds copper ions *in vitro*. To investigate whether DHLA can remove metal ions from the active site of enzymes, DHLA was incubated with CuZn SOD and aconitase. Diethyldithiocarbamate (DDC), which inactivate CuZn-SOD by removing copper was used as a positive control. In addition, tetraethylthiuram disulfide (TTD), the oxidized form of DDC was also used to compare its effects to those of LA. As shown in Figure 3.2, DHLA, LA, or TTD did not alter SOD activity, whereas DDC inhibited SOD activity in a concentration dependent manner (34% and 80% inhibition with 0.1 and 0.5 mM DDC, respectively;  $p < 0.0001$ ; Figure 3.2). These results show that although both DHLA can chelate free copper ions in a redox-inactive manner, DHLA does not inactivate CuZn SOD by removal of the active-site copper ion.

To study the effects of LA and DHLA removal of iron ions from enzymes, the porcine heart aconitase was used. Aconitase contains a labile iron core, which is required for its activity. As shown in Figure 3.3, aconitase activity was 70% reduced ( $p < 0.001$ ) in the presence of EDTA, used as a control for LA and DHLA. In contrast, no significant changes in aconitase activity were observed with 0.2-2 mM LA or DHLA (Figure 3.3).



**Figure 3.2. Only DDC, but not DHLA, LA or TTD inactivate Cu,Zn-SOD.**

The ability of both oxidized and reduced DDC and DHLA to remove Cu(II) from Cu,Zn-SOD was examined. Briefly, SOD (2  $\mu$ g) was incubated in the presence of either 0.1 or 0.5 mM DDC, TTD, DHLA, or LA for 1 hour. The resulting changes in SOD activity were determined by monitoring the rate of pyrogallol autoxidation. DDC, a known thiol inhibitor of SOD, decreased SOD activity by copper removal. In contrast, LA and DHLA did not inhibit SOD activity, suggesting that no copper was removed from SOD. Results are expressed as mean  $\pm$  SD. (\* denotes statistically significant differences from controls)



**Figure 3.3. LA and DHLA does not remove Fe(II) from aconitase.**

The ability of LA and DHLA to remove Fe(II) from aconitase was examined. Briefly, 0.25 mg of aconitase was incubated in the presence of either LA (0.2, 0.5, 1. and 2 mM) or DHLA (0.5,1,2,3, and 5 mM) for 30 minutes. The resulting changes in aconitase activities were determined as described in methods. While chelation by EDTA caused the inactivation of aconitase activity, no apparent changes in aconitase activities were observed with LA and DHLA. Results are expressed as mean $\pm$ SD. (\* denotes statistically significant differences from controls)

### 3.5 Discussion

In this study, the effects of LA and DHLA on the redox activity of iron and copper complexes were examined. In addition, we also assessed whether LA and DHLA can remove enzyme-bound metal ions. Results show that chelation of iron and copper by DHLA caused a decline in the redox activities of these metals. In contrast, LA was not effective in modulating the redox activities of either iron or copper ions. Despite slowing metal-mediated ascorbate oxidation, DHLA, as well as LA, were not effective in chelating iron and copper bound to aconitase and Cu,Zn-SOD, respectively. These results suggest that DHLA protects against metal-induced oxidant injury without removing the metal ions from the active sites of metalloproteins.

The reduction in the rate of Fe(III)-citrate-mediated ascorbate oxidation by DHLA is consistent with the observation that DHLA forms a stable complex with Fe(III) [58,106]. While DHLA only slightly inhibits iron-mediated ascorbate oxidation, it very effectively lowered the rate of copper-mediated ascorbate oxidation, suggesting that DHLA chelates copper more effectively than histidine.

CuZn SODs exists as dimers of identical subunits, with each subunit containing a catalytic copper ion bound to four histidine residues [107]. Substitution or loss of copper results in a complete loss of catalytic activity, while Zn can be replaced by several cations. The entrance to the active site of SOD is about 4 Å wide and allows for small chelators such as DDC to efficiently remove copper [108]. The S-S bond length of the 1,2-dithiolane ring of LA is estimated to be 2.05 Å and thus, LA is

not limited by its size to gain access to copper in the active site [52]. Although DHLA effectively inhibited Cu(II)-histidine-mediated ascorbate oxidation, it did not remove histidine-stabilized copper located within the active site of SOD.

The active site of aconitase contain a four labile iron atoms that are coordinated by four cyteine residues, and is sensitive to removal by chelators or under conditions of iron depletion [21]. The cytosolic isoform of aconitase also act as the cellular iron sensor since the incorporation of iron to form iron-sulfer center is highly dependant on the cellular iron levels [21]. Because of the labile nature of iron in its active site, aconitase has been successfully used as an assay system to detect free labile iron in biological fluids such as serum [105]. For the same reason, aconitase also serves as an excellent test system to determine whether a given compound can remove metals from proteins. Our observations that both DHLA and LA are not able to remove iron from aconitase and reduce aconitase activity suggest that it is highly unlikely that these compounds cause iron depletion in cells.

In summary, DHLA may serve as a novel agent to lower toxicities associated with redox-active transition metal-ions [109]. Unlike other thiol agents, LA, which is rapidly reduced *in vivo* to DHLA, appears to be well tolerated in humans even at very high doses of up to 600 mg/day [102]. In addition, its rapid absorption and uptake into tissues such as the brain and subsequent reduction to DHLA, makes LA an ideal alternative for the treatment of metal-dependent oxidative stress. Further studies are needed to test the physiological relevance of the chelating properties of DHLA.

### **3.6 Acknowledgement**

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## **Chapter 4**

**Dietary supplementation with (*R*)- $\alpha$ -lipoic acid reverses the age-related accumulation of iron and the depletion of antioxidant status in the brain**

Jung Suh, Regis Moreau, Shi-Hua Heath and Tory M. Hagen

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#### 4.1 Abstract

(*R*)- $\alpha$ -Lipoic acid (LA) is a dithiol precursor to the lipoamide prosthetic group in  $\alpha$ -keto acid dehydrogenases, which is critical for both carbohydrate and lipid catabolism. Exogenously supplied LA is readily taken up by cells and reduced to dihydrolipoic acid (DHLA), which exhibit potent antioxidant activities, including the ability to chelate transition metal-ions (such as iron) and to regenerate other low molecular weight antioxidants (such as ascorbate and glutathione). Given that the aging brain exhibits an excessive iron accumulation and compromised tissue antioxidant status, we investigated whether supplementing the diets of old rats with LA (0.2% w/w) would result in improvements in these parameters. Results show that the tissue iron levels in cerebral cortex obtained from old rats supplemented with LA were significantly lower when compared to their age-matched controls. In fact, iron levels in old rats which otherwise increased by approximately 80% were normalized to levels that were no longer significantly different from young control rats. Additionally, LA supplementation to old rats improved the age-dependent declines in both ascorbate and glutathione (GSH) levels, which declined by 28% and 27%, respectively with age. LA supplementation also attenuated the decline in GSH/GSSG ratio, a key indicator of cellular redox state, which also declines by 55% in the aging rat brain. Correlations of iron levels to tissue ascorbate, GSH and GSH/GSSG ratios showed a significant inverse relationship with tissue GSH and GSH/GSSG ratios but not with ascorbate. The significant inverse correlation between tissue iron and thiol status provides indirect

evidence for iron as a significant contributor to the age-associated increase in oxidative stress in the brain.

## 4.2 Introduction

Chronic oxidative stress is a major factor contributing to brain senescence, as well as part of the etiology of age-associated neuropathologies, such as Parkinson's and Alzheimer's diseases. Numerous indices of oxidative damage such as lipofuscin [24], malondialdehyde [110], protein carbonyls [111] and oxo<sup>8</sup>-dG [112] increase as a function of age and their accumulation strongly correlates with declines in cognitive functions. Although the causal factors responsible for the age-related increases in oxidative damage are not completely understood, there is a general consensus that heightened accumulation of transition metals, (e.g. iron and copper) and decrease of intracellular antioxidants contribute significantly to the pro-oxidant environment of the aging brain [29,113,114].

In particular, iron can be a double-edged sword for the cell. Although iron is required for normal neuronal functions, when levels exceed cellular binding capacity, iron can promote hydroxyl radical formation, leading to oxidative damage and resultant cellular decay. The latter scenario appears to occur in the aging brain as iron levels in specific regions such as the basal ganglia far exceed the normal metabolic requirement for iron [22,23,98,115]. While numerous studies implicate pro-oxidant role of iron in the aging brain, the extent and the locality of brain iron deposition is not clear. In this regard, mitochondria may be an ideal intracellular locale for iron deposition because they are the major intracellular organelle responsible for the synthesis of heme and iron-sulfur clusters and thus are the principal organelle for iron metabolism and disposition [21,116-118]. However, despite their central role in iron

metabolism, whether mitochondria preferentially accumulate iron during aging has not been examined.

Aside from increased iron deposition, certain cellular antioxidant defenses decline with age. Most notably, the important water-soluble antioxidants, ascorbate (AA) and glutathione (GSH), decline significantly in the aging rat brain [119,120]. In non-pathological adult brain, ascorbate and glutathione are present in millimolar concentrations. Their distribution within the neuronal cell types show marked differences with preferential accumulation of AA in neurons and GSH in glial cells [121,122]. Thus, the combined loss of both AA and GSH suggest a global attenuation of low molecular weight antioxidant defense that are especially required to protect against iron-catalyzed free radicals.

Based on these studies, it appears that therapeutic interventions to lower the age-related accumulation of iron and/or restore GSH and AA levels would be beneficial to maintain normal redox balance and cognitive function. However, treatments with conventional iron chelators, such as desferrioxamine, are not effective because of their inability efficiently cross the blood brain barrier [100]. Thus, effective modulation brain iron status requires the use of large amounts of chelators, which can be extremely toxic. Antioxidants such as ascorbate or  $\alpha$ -tocopherol that can readily cross the blood brain barrier do not appear to accumulate globally or affect iron status or most importantly actually lower parameters of heighten oxidative insult seen during aging. Thus, there is a need for finding new means to improve both alterations in iron and antioxidant status that is also non-toxic.

In this regard, (*R*)- $\alpha$ -Lipoic acid (LA), a compound exhibiting both metal chelating and antioxidant properties might be a useful pharmacotherapy to lower the age-associated increase in oxidative stress of the brain. LA is a naturally occurring dithiol compound that is normally bound as a lipoamide prosthetic group present in  $\alpha$ -keto acid dehydrogenases [46]. However, free LA readily crosses the brain brain barrier and is rapidly taken up and reduced to dihydrolipoic acid (DHLA) in both neurons and glial cells. DHLA exhibits potent antioxidant activities and can directly terminate a variety of reactive oxygen and nitrogen species; moreover, because of the low reduction potential of LA/DHLA redox couple (-320 mV), it has been shown to reduced other endogenous antioxidants, including ascorbate and GSH [102]. We have noted that feeding old rats LA also restores the age-associated declines in hepatic and myocardial AA and GSH, which, like the brain, declines with age. It's antioxidant potential is augmented by its ability to bind transition metals, such as iron and copper, so that these transition metals can no longer participate in metal-catalyzed free radical reactions (Suh et al., manuscript in preparation). We thus hypothesized that supplementing the diets of old rats with LA could improve cellular redox balance which otherwise becomes perturbed with age.

Thus, the aims of this study were to examine: 1) the extent and locality of iron accumulated in the aging brain and how these changes may correlate with endogenous antioxidants such as AA and GSH 2) whether dietary supplementation with LA modulate the age-dependent compromise in the cerebral iron and antioxidant status. Our results indicate that feeding LA to old rats for two weeks effectively reverses the

age-related changes increase in iron and restores the age-associated depletion of AA and GSH levels in the aging brain.

### 4.3 Materials and methods

#### *Materials*

The following chemicals were used: mannitol, sucrose, diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), Tris-base, Percoll, L-ascorbic acid, perchloric acid, GSH, oxidized glutathione (GSSG), heparin (sodium salt), and fatty acid free bovine serum albumin (Sigma, St. Louis, MO); Chelex-100 resin (BioRad Technology, Hercules, CA ); inductively coupled plasma-mass spectroscopy (ICP-MS)-grade iron standards (Fisher Scientific); All other reagents were reagent grade or better.

#### *Animals*

Rats (Fischer 344, virgin male, outbred albino; National Institute of Aging animal colonies), both young (3 mo; N=8) and old (24 to 28 mo; N=8) were acclimatized in the Oregon State University animal facilities for at least 1 week prior to experimentation. Animals were placed on an AIN-93M standard diet (Dyets Inc., Bethelhem, PA); LA-fed rats were given AIN-93M diet supplemented with 0.2% (w/w) (*R*)- $\alpha$ -lipoic acid (AstaMedica, Germany) for two weeks prior to sacrifice. Animals had free acces to food and water throughout the trial. Food consumption did not differ between age groups or treatments (young control:  $21.0 \pm 0.4$ , young LA:  $18.0 \pm 2.3$ , old control:  $20.9 \pm 0.8$ , and old LA:  $18.7 \pm 0.8$  g of food consumed/day).

Following two-weeks of supplementation, rats were anesthetized with diethyl ether and a midline incision was made in the abdomen. Heparin (0.4 mg/ml) was injected via the femoral vein and blood was removed by retrograde perfusion with Hank's balanced salt buffer (pH 7.4) for 5 min via the thoracic aorta and let the blood out by cutting the posterior Vena Cava above the renal vein. Brains were removed and rinsed in ice-cold chelex-treated phosphate buffered saline (PBS; pH 7.4). Brains were divided into left and right hemispheres and the cerebral cortex isolated from each section. For experiments measuring antioxidant levels, the cortical material obtained from the left hemisphere was homogenized immediately in perchloric acid (10% w/v) containing 5 mM DTPA and were snap-frozen in liquid nitrogen. For the assessment of total cerebrocortical iron content, cortical samples from the right hemisphere were dehydrated overnight in acid-washed glass tubes at 90 °C in a drying oven.

#### *Mitochondrial isolation*

Mitochondria were isolated according to the method of Anderson and Sims [123]. Briefly, freshly isolated brains were minced in ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EDTA and 10 mM Tris; pH 7.4. Minced tissue samples (10% w/v) were homogenized with a loose-fitting Dounce homogenizer, followed by additional homogenization with a tight-fitting Dounce homogenizer. The crude homogenates were transferred to 15 ml centrifuge tubes and diluted with an equal volume of 24% (v/v) Percoll. These samples were centrifuged at  $31,000 \times g$  for 15 min at 4°C. The top layer (15 ml) was removed and the volume

readjusted to 30 ml using Percoll (14% [v/v]), rehomogenized using a tight fitting Dounce homogenizer and centrifuged at  $31,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The top layer (15 ml) was discarded and the resulting bottom layers were combined. Digitonin (200  $\mu\text{l}$ ; 50 mg/ml in water) was added, mixed gently and layered onto a Percoll gradient solution (40% and 19% Percoll:water solutions; 10 ml each) and centrifuged at  $31,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The enriched mitochondrial fraction was removed from the interface between 40% and 19% Percoll solutions and further centrifuged at  $16,700 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatants were discarded and the mitochondrial pellets were resuspended in isolation buffer (200  $\mu\text{l}$ ). The protein yield was quantified using a standard Lowry assay with BSA as the external standard. Mitochondrial protein yields from young and old rats were similar ( $3.8 \pm 0.8$  mg protein/ml vs  $3.2 \pm 1.2$  mg protein/ml; young and old rats, respectively).

#### *Ascorbic acid analysis*

Ascorbate was measured as described previously [62]. Briefly, acidified tissue homogenates were centrifuged to remove precipitated proteins. Ascorbic acid was separated by high performance liquid chromatography (HPLC) [62] and detected using a LC 4B electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN) at an applied potential of +0.6 V.

### *GSH analysis*

GSH levels in the cerebrocortex were measured according to the method of Jones *et al* [124]. Briefly, tissue samples were centrifuged to remove the precipitated proteins.  $\gamma$ -Glutamyl-glutamate (10  $\mu$ M; internal standard) and iodoacetic acid (7.4 mg/ml in water) as added to 150  $\mu$ l of the perchloric acid extracts and the pH adjusted to 9.0. These samples were incubated at room temperature for 20 min in the dark. Following the treatment with iodoacetic acid, dansyl chloride (20 mg/ml in acetone) was then added and incubated overnight in the dark at room temperature. These samples were extracted with 500  $\mu$ l of chloroform and were separated by HPLC using a 3-aminopropyl spherisorb column (Cell Associates) and detected using a Hitachi FL-6000 fluorescence spectrophotometer with monochromators set at 335 nm for excitation and 515 nm for emission. Quantitation was obtained by integration relative to internal and external standards.

### *Iron analysis*

For the analysis of total tissue iron content, dried tissue samples were weighed and acid digested in 50% HNO<sub>3</sub> (w/v in water) at 80 °C for 6 hrs. Following acid digestion, samples were diluted to a final HNO<sub>3</sub> concentration of 5% (w/v in water). For the analysis of mitochondrial iron content, mitochondria (200  $\mu$ l; 5 mg/ml protein) were mixed with 5% (w/v) HNO<sub>3</sub> and incubated at 80 °C for 1 hr. Iron content in these samples was measured by using inductively coupled plasma atomic emission

spectroscopy (ICP-AES; Varian Liberty 150). Atomic emission profile of iron was monitored at 259 nm and authenticated relative to standards.

### *Iron efflux studies*

LA-induced efflux of brain iron was investigated in old rats (27-28 mo) fed AIN-93M diet for two weeks. Fresh cerebral cortex was isolated and sub-divided into left (control) and right (LA) hemispheres. These samples were hand sliced to approximately 1 mm in thickness and were incubated in chelex-treated oxygenated Krebs-Henselheit buffer (0.5 mM glucose; pH 7.4) in the presence or absence of 1 mM LA for one hour at 37°C. Aliquots of the supernatant were collected at time 0 and following 1 hr; iron released into the media determined by ICP-AES. The iron levels were also determined in these tissue samples following incubation by ICP-AES and total tissue iron levels were defined as the sum of iron present in the supernatant and in tissue slice samples. Results were expressed as the % of total iron released into the media. Oxygen consumption of the brain sections measured using a Clark-Type oxygen electrode (Yellow Spring, OH) was used as an indicator of tissue viability.

### *Statistical Analysis*

Statistical significance was determined by the one way - analysis of variance (ANOVA) using a Statview statistical software. Tukeys post-hoc test was used to determine differences between treatment groups. Results are expressed as the mean  $\pm$  SEM or SD. A P-value of less than 0.05 was considered significant.

#### 4.4 Results

##### *Age-related changes in brain iron levels in the tissues and mitochondria*

To determine the extent and the subcellular locale of iron accumulation in young versus old rat brains, iron levels in the cerebral cortex were determined by ICP-AES. Total cortical iron levels increased from  $87.25 \pm 24.2$  ng iron/mg dry tissue weight in young rats to  $156.86 \pm 20.59$  ng iron/mg dry tissue weight in 28 mo animals, a significant 80% ( $p < 0.01$ ) increase over young controls (Figure 4.1A).

To establish whether this age-associated elevation in cortical iron levels was due to aberrant accumulation in mitochondria, the extent of age-related changes in mitochondrial iron levels was determined. Results show that, despite a pronounced increase in total cortical iron levels, mitochondrial iron levels remained unchanged in young versus old animals ( $0.49 \pm 0.1$  ng iron/mg protein versus  $0.55 \pm 0.12$  ng iron/mg protein; young versus old, respectively; Figure 4.1B). Thus, the age-related accumulation of iron was extra-mitochondria in nature.

##### *Age-related changes in low molecular weight antioxidants in the brain*

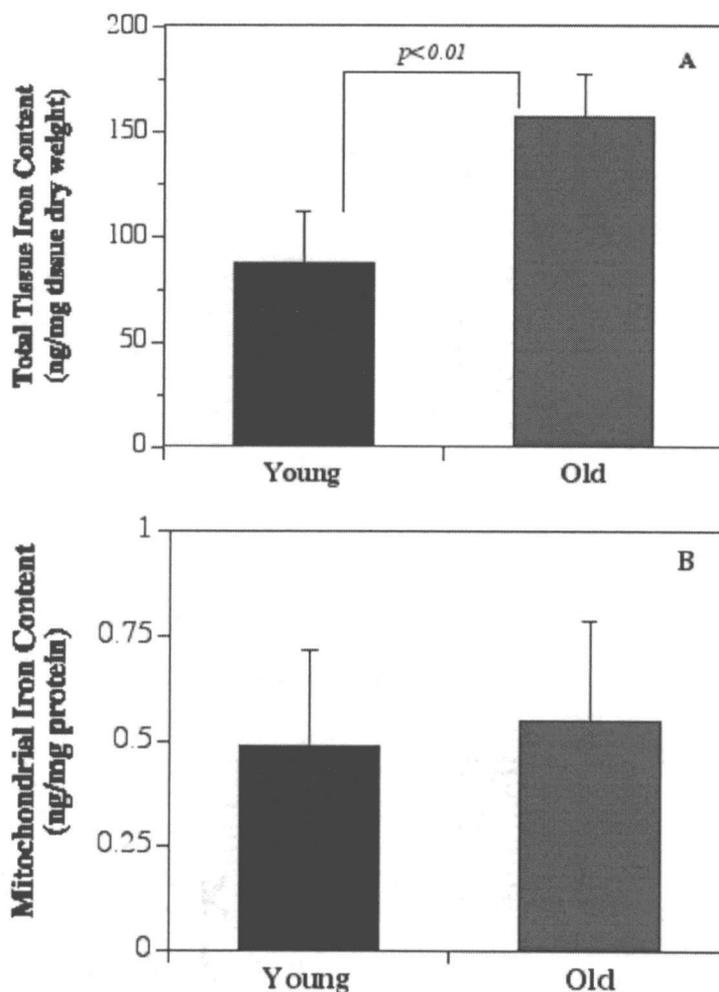
Because iron accumulation suggests that conditions exist for oxidative stress in the aging rat cerebral cortex, AA and GSH, antioxidants that are directly involved in maintaining neuronal cell redox balance were determined to gauge the overall antioxidant potential to limit redox changes in cortex with age. Analysis of cerebral AA levels shows that cortical AA are significantly lower (27%) in old compared to

young rats (Figure 4.2A). Because AA has been found to preferentially accumulate in neurons, its loss suggests that neuronal antioxidant defenses are compromised with age.

In contrast to AA, higher concentrations of GSH are synthesized and maintained in the glial cells [121]. Because glial cells, such as oligodendrocytes are the main cell types involved in iron accumulation [125], we examined the extent of age-related changes in GSH levels. Results show that total GSH (GSH+GSSG) declined by approximately 25% ( $p < 0.02$ ) in old when compared to young rat (Figure 2B). The decrease in the total GSH was accompanied by the perturbations in GSH/GSSG ratios, resulting in an almost 70% ( $p < 0.0001$ ) decline in the GSH/GSSG ratio with age (Figure 2C). The significant alterations in GSH:GSSG ratios along with depletion of GSH levels suggest that cerebral thiol redox status declines with age.

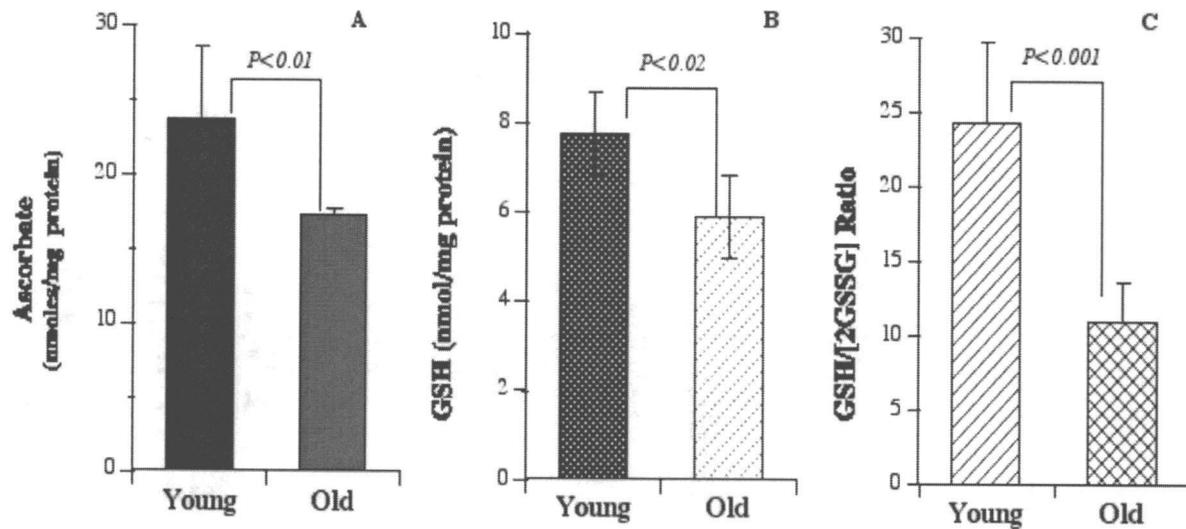
#### *Effects of dietary supplementation with lipoic acid on brain iron status*

We previously showed that feeding old rats diets supplemented with LA restored hepatic and myocardial AA levels and GSH redox state, while others *in vitro* show that LA is an effective metal chelator. To determine whether LA could improve low molecular weight antioxidant status and/or decrease the age-related increase in iron levels, young and old rats were fed 0.2% LA for two-weeks and the changes in the cerebral iron, AA, GSH and GSH/GSSG ratios were assessed. LA supplementation even at this pharmacological dose did not appear to cause any untoward side-effects in



**Figure 4.1.** Aging results in a significant increase in total iron levels but mitochondrial iron pool does not change with age.

The iron concentrations in total tissue homogenate (A) and in mitochondria (B) obtained from young (N=5) and old (N=4) were examined. The results show that the total iron content in the whole brain homogenate increased in old animals ( $0.15 \pm 0.01$  ng/mg protein) when compared to young animals ( $0.1 \pm 0.01$  ng/mg protein). Despite this heightened iron accumulation, the total iron content did not increase in mitochondria isolated from old versus young animals ( $0.5 \pm 0.1$  versus  $0.55 \pm 0.11$  ng iron/mg protein; young and old respectively). Results are expressed as Mean  $\pm$  SEM.



**Figure 4.2. Cerebral ascorbate, GSH and GSH/GSSG ratios decline significantly in the senescent rats.**

The cerebral ascorbate (panel A) and GSH levels (panel B) from young and old rats were measured as described in the methods. The results show a significant decline in the ascorbate and reduced GSH levels. GSH/GSSG ratios (panel C), a key parameter of thiol redox status decreased in the aging rat brain. The results are expressed as mean  $\pm$  SEM.

either young or old rats throughout the feeding study. LA supplementation also did not affect cortical iron status in young rats (Figure 4.3A). Similarly, the mitochondrial iron levels in young rats were not affected by LA supplementation (Figure 4.3B). These results suggest that in young adult rat brain with normal iron status, LA had no modulatory effects on iron levels.

In contrast to young animals, old rats on the LA supplemented diet exhibited a significant attenuation of the age-dependent increase in total tissue iron levels ( $0.09 \pm 0.01$  ng/mg protein versus  $0.13 \pm 0.01$  ng/mg protein; old LA versus old control, respectively;  $p < 0.012$ ; Figure 4.3A). Iron levels in LA supplemented old rats were no longer significantly different from young rats either on the LA or the control diets. These results suggest that LA only affected the elevation iron pool that occurred with age but did not otherwise affect iron status associated with normal brain function. As shown in Figure 4.3B, no significant changes in mitochondrial iron content were observed between controls and LA supplemented old rats. These results suggest that LA feeding does not effectively remove iron from mitochondrial, and the excess iron is being removed from extra-mitochondrial source.

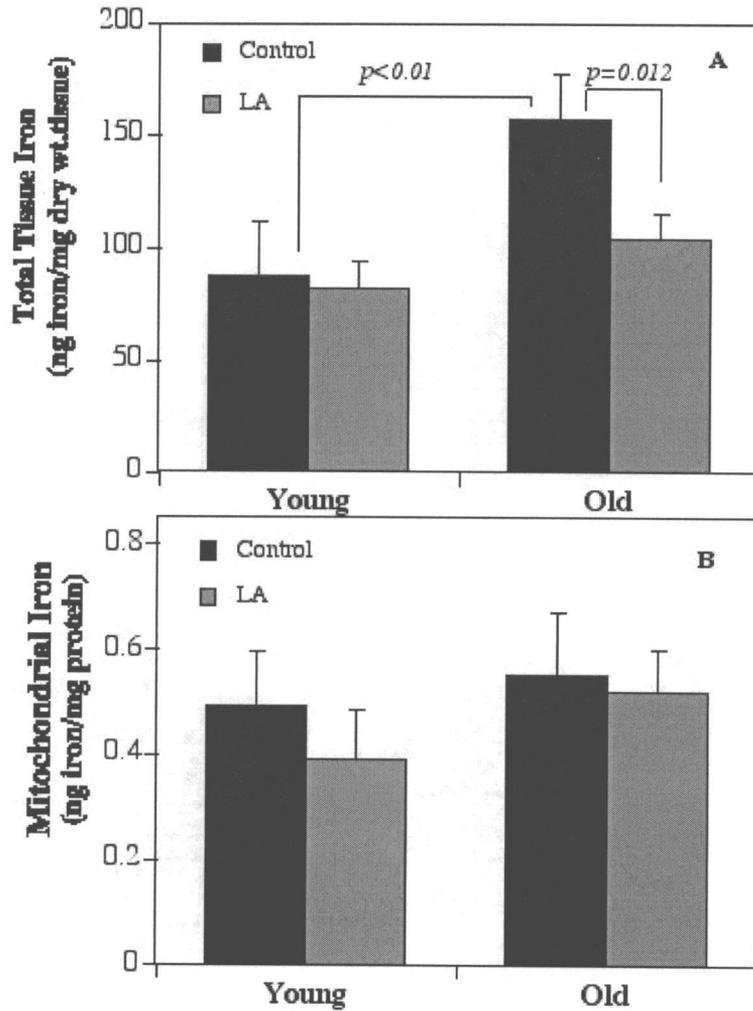
LA is rapidly taken up, reduced and effluxed as DHLA in cell culture systems, we tested the possibility that LA may remove iron during this rapid efflux process. To do this, an in vitro model system using cortical tissue slices was established whereby the rate of iron release could be monitored following addition of LA. Freshly isolated cortex from young and old rats was incubated in the presence and absence of 1 mM LA for up to one hour. To control for the changes in cell viability during the

experiment, the rate of oxygen consumptions were monitored. No apparent changes in the rate of oxygen consumption were observed (data not shown). As shown in Figure 4.4, the amount of iron released in control and LA-treated cortical slices obtained from old rats were virtually identical. These results suggest that the restoration of normal iron levels over the two-week feeding regimen may not be due to the rapid efflux of an iron:DHLA complex.

#### *Effects of LA supplementation on brain antioxidant status*

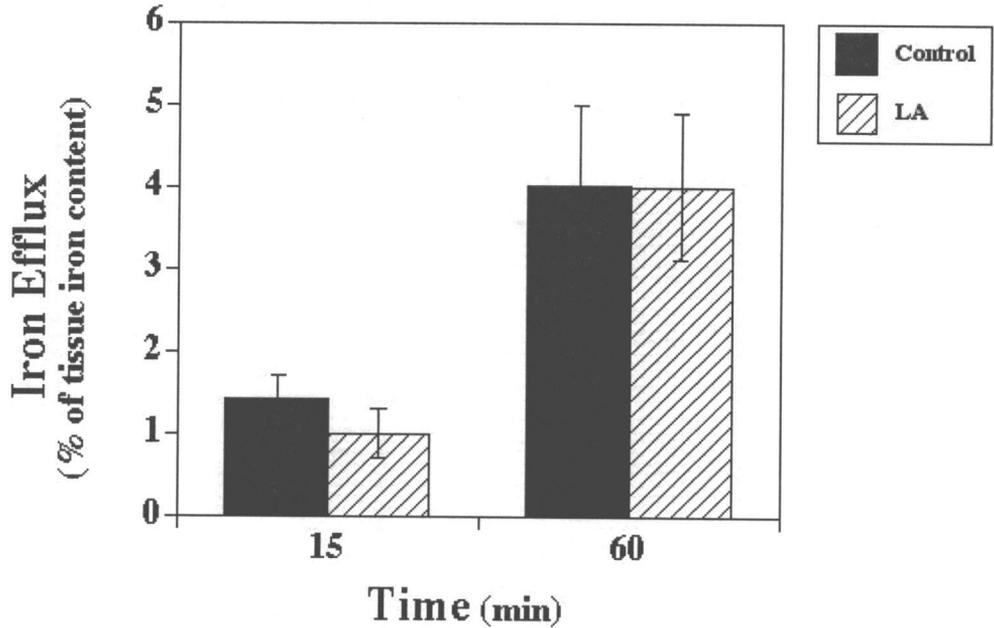
To determine whether feeding LA also resulted in restoration of cortical antioxidant status and thiol redox ratio, rats both young and old were fed with or without LA and resulting changes in antioxidant status determined. The changes in cerebral AA status in young rats fed either control or LA-supplemented diet is shown in Figure 5A. LA feeding did not further increase the tissue AA levels beyond what was typically observed in young rats fed control diets (Figure 4.5A). Similarly, no significant LA-dependent changes in either GSH (Figure 4.5B) or GSH/GSSG ratios (Figure 4.5C) were observed between young control- and LA-fed rats (Figure 4.5). These results are consistent with results obtained in our previous studies showing that LA does not enhance tissue antioxidant status beyond the normal levels found in young unsupplemented animals

In old rats, however, LA supplementation significantly improved tissue AA status, which effectively increased to levels such that they were no longer significantly



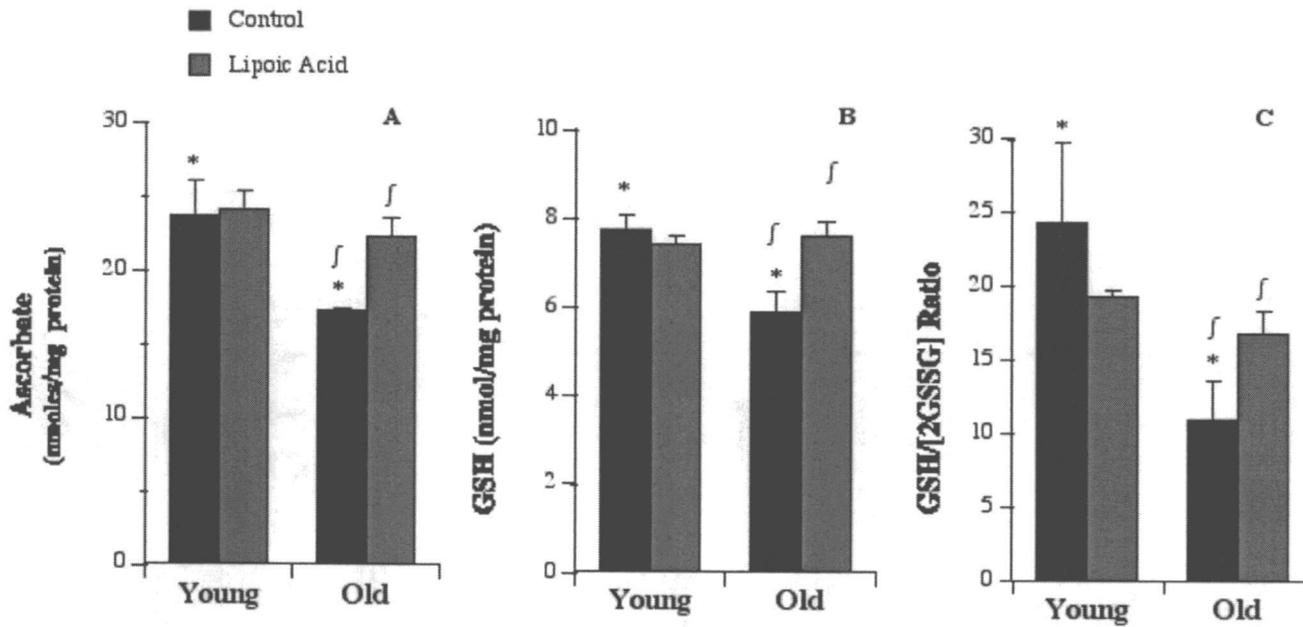
**Figure 4.3. LA supplementation reverses the age-related increase in iron levels.**

Iron levels in freshly isolated brain from old (N=8) and young animals (N=8) were determined following a two week supplementation of AIN-93M diet with or without LA. Results show that only in old rats, LA feeding lowered the age-related increase in cortical iron content, while iron levels in young rats remained unchanged (Figure 3A). In both young and old animals, mitochondrial iron content were unaffected by the dietary LA supplementation (Figure 3B). Results are expressed as Mean  $\pm$  SEM



**Figure 4.4. LA does not remove iron by direct efflux mechanism.**

The Frontal cortex was isolated from old rats (N=4) fed AIN-93M diet for two weeks. The isolated cortex was sub-divided into left (control) and right (LA) hemisphere and was sliced into 1 mm thickness. These slices were incubated in Krebs-Henseleit (0.5 mM glucose; pH 7.4) in the presence or absence of 1 mM LA for up to one hour. The amount of iron effluxed from the tissues was measured by using ICP-MS. The results are expressed as % total tissue iron content (sum of effluxed iron + tissue iron content; 100% value =  $10.5 \pm 1.7$  ng/mg tissue wet wt.).



**Figure 4.5. LA supplementation restores the age-related decline in ascorbate status and restores age-dependent decline in GSH to GSSG ratio in the brain.**

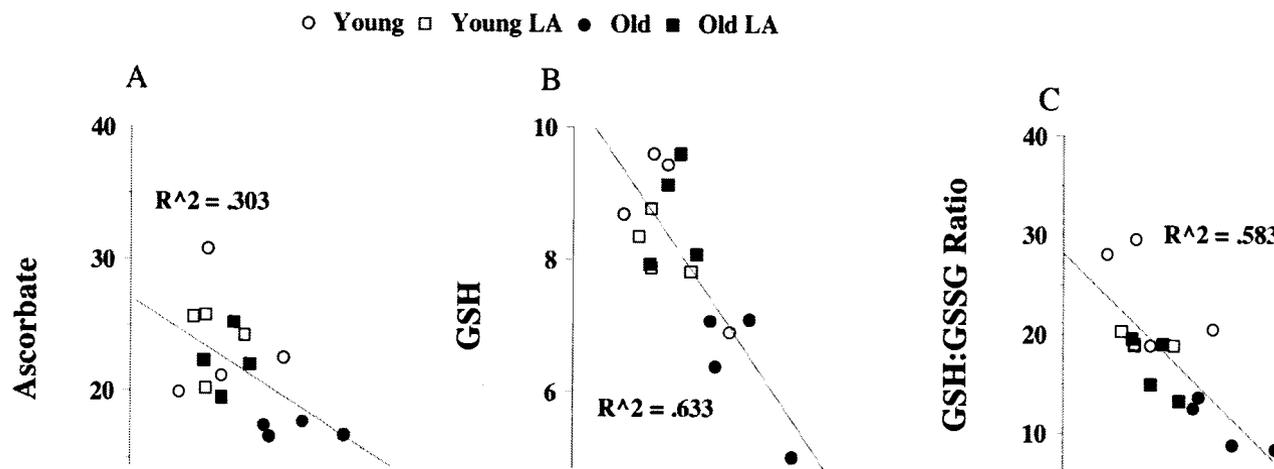
Ascorbate, GSH, and GSSG levels in freshly isolated brain from old (N=4) and young (N=4) rats following a two-week supplementation with AIN-93M diet with or without 0.2% (w/w) (R)-a-lipoic acid. The ascorbate and GSH to GSSG ratio in the old supplemented animals was significantly higher than old unsupplemented animals. Results are expressed as mean  $\pm$  SEM.

different from young animals (Figure 4.5A). Likewise, the age-associated declines in tissue GSH levels were also alleviated by LA supplementation (Figure 4.5B), leading to a significant improvement in GSH:GSSG ratio ( $p < 0.05$ ; Figure 4.5c). These results show that LA improves the age-related decline in the brain antioxidant status.

*Correlation between changes in cerebral iron and ascorbate, GSH and GSH redox state.*

Because the mean values for iron and antioxidant status show an apparent inverse relationship, the correlation between individual iron levels and ascorbate, GSH and GSH redox state were determined. Tissue iron levels were showed a significant inverse correlation with tissue GSH redox state with  $R^2$  values of 0.59 ( $P < 0.0005$ ; Figure 4.6C). The inverse relationship between iron and GSH redox state was conserved even when analysis was performed within either young or old animals, suggesting a close link between iron accumulation with perturbations in thiol redox status. This relationship is also augmented by the fact that a strong inverse correlation between iron and total GSH were observed with  $R^2$  value of 0.66 ( $p < 0.0005$ ; Figure 4.6B).

With respect to ascorbate, we observed a small but significant inverse correlation with  $R^2$  values of 0.30 ( $p < 0.001$ ; Figure 4.6A). However, when correlation analysis was performed within young or old age groups, no inverse correlation between iron and ascorbate was present. These results indicate that the age-associated loss of ascorbate seen appears to be independent of changes in cortical iron status.



**Figure 4.6. Increased in cortical iron levels inversely correlates with tissue GSH status**

The correlation between cerebral iron levels and ascorbe, GSH, and GSH/GSSG ratios were determined by linear regression. The age-dependent increase in iron levels showed a weak inverse correlation with tissue ascorbate status with  $R^2$  values of 0.33. However when adjusted for age, the inverse correlation between iron and ascorbate was no longer significant (Panel A). The correlation between cortical iron levels and total GSH levels are shown in Panel B. Results show a strong inverse correlation with  $R^2$  value of 0.66, indicating that iron significantly influences tissue GSH levels. Similar to total GSH, cortical iron levels strongly correlated with the loss in cerebral GSH:GSSG ratios (Panel C).

## 4.5 Discussion

This study shows that there is a significant age-dependent deposition of elemental iron in the cerebral cortex of rats. This is consistent with previous studies that show accumulation of iron in this region as well as other reports indicating that iron levels also accumulate in specific subregions of the cortex, such as the hippocampus and striatum [113,115,125]. Deposition of often substantial levels of transition metal ions in these regions, such as is also considered part of the etiology of age-related neurological disorders like Parkinson's and Alzheimer's diseases. Therefore, iron accumulation correlates with heightened risk for loss of cognitive function, which depending on the degree of accumulation, can be mild or severe.

The mechanism(s) as to how iron participates in the pathologies associated with brain aging are believed to be through its role in reactive oxygen and nitrogen species production. Iron is recognized as a potential pro-oxidant and can participate, via Fenton chemistry, in the production of free radicals. Indeed, biomarkers of oxidative damage such as 8-oxo-2'-deoxyguanosine and measures of lipid peroxidation, increase concomitantly with iron deposition. Heightened iron in the aging brain has also been associated with oxidation of catecholamines to neurotoxic semiquinone metabolites capable of producing significant quantities of oxidants, which have been shown to induce neuron loss and attendant cognitive decline. In the present study, we found a striking inverse correlation between iron and brain GSH/GGS ratio, a major thiol redox couple that has been used as a measure of overall redox state of the cell. This tight association between GSH redox state and iron again

strongly suggests that increased tissue iron status adversely affects the pro- versus anti-oxidant balance, which confirms that increased iron associated with aging has severe consequences in terms of oxidative stress.

Mitochondria, whose function declines appreciably with age and are also the major site for heme and iron-sulfur cluster biosynthesis, would be hypothesized to be a major site for iron accumulation. Contrary to expectation, we did not observe significant differences in mitochondrial iron levels between preparations isolated from old versus young rats. It must be noted, however, that this apparent lack of mitochondrial involvement may also be due to loss of the most compromised mitochondria upon isolation or due to sequestration of iron-laden mitochondria into phagolysosomal bodies, which would not be expected to be isolated using the procedure employed in this study. Despite these caveats, our data suggests that actively respiring mitochondria from aging rat neocortex do not accumulate appreciable amounts of iron and thus the observed age-related increases in iron are largely of extra-mitochondrial origin.

Taken together, our evidence confirms that the simultaneous accumulation of iron and loss of low molecular weight antioxidants in the aging rat brain shifts the redox balance towards a more pro-oxidant state. Thus regimens to limit iron accumulation and/or restore tissue antioxidant status should be beneficial in attenuating oxidative stress and thereby improving cognitive function. To this end, our work and that of others, indicates that LA may be a particularly suitable pharmacologic agent to restore redox balance both by attenuating the increase in iron

levels and improving antioxidant status in the aging brain. Unlike most metal chelators, LA readily crosses the blood brain barrier and accumulates in all regions of the central nervous system and peripheral nerves [102]. Moreover, exogenously supplied LA is rapidly reduced to DHLA, which binds with iron and copper and chelates those metals in a redox inactive manner [101]. Also, LA does not appear to cause potentially toxic side-effects by removing metals from iron-containing proteins (Suh et. al., manuscript in preparation). This last property was recently demonstrated when we found that even high levels of LA or DHLA could not remove iron from aconitase or SOD. In the present work, we also found that LA modulated only the *increase* in iron content in aging animals and did not affect either the normal homeostatic levels in young rats or lower iron status in old rats below that seen in young animals. This important property supports the view that LA supplementation does not cause iron depletion, but restores normal iron status that otherwise increases with age.

The observed increase in cytosolic iron content may be a reflection of deposition of aging pigments, such as lipofuscin [24]. Lipofuscin is a complex, insoluble intracellular pigment that is characterized to contain polymerized residues of oxidized lipids and proteins [25]. In experimental animals, lipofuscin has been shown to increase linearly with age in neurons throughout the central nervous system [24-27]. In addition, there is also a progressive accumulation of neuromelanin with aging [28]. Neuromelanin is characterized by deposition of polymerized dopamine oxidation products. In aging, neuromelanin has been shown to accumulated in the brain stem

nuclei (substantia nigra, locus coeruleus, and dorsal motor nucleus of vagus nerve) [28]. Neuromelanin has high binding affinity for iron and has been demonstrated to be the major iron storage protein in the substantia nigra region of the brain [29]. Under normal conditions, the chelation of iron by neuromelanin may be protective but during conditions of the iron overload, the iron binding sites may become saturated, leading to increased availability of free redox active iron. The saturation of iron loosely bound to “aging pigments” may potentially increase oxidant load and result in the observed increased rate of antioxidant consumption.

In addition to attenuating the age-associated increase in iron levels, LA supplementation also reversed the decline in tissue ascorbate and GSH levels and restored the GSH/GSSG ratio. Several mechanism(s) may be involved in this improved antioxidant status. First, because of the low reduction potential of the DHLA/LA redox couple, other antioxidants, such as ascorbate and GSH, could be recycled and thus LA treatment could spare these endogenous antioxidants from oxidation and removal. Second, LA may actually increase overall cellular reductive capacity by increasing NADPH levels, thereby providing increased reducing power for cellular antioxidant enzymes [126,127]. Finally, LA may induce cellular GSH synthetic capacity by increasing the cellular uptake of cysteine, which is the limiting substrate for GSH biosynthesis [128]. Thus, further work will be necessary to determine the precise mechanism(s) related to the pharmacologic action of LA in improving antioxidant capacity in the aging brain.

In summary, the current study shows that LA provide a novel and promising therapeutic approach for the prevention and treatment of age-related neurodegenerative diseases. Further work, however, will be required to determine the precise mechanisms of action, proper dosage, pharmacokinetics and potential detrimental side-effects of this compound.

#### **4.6 Acknowledgement**

This work was supported by National Institute of Aging Grant RIAG17141A (T.M.H.),

**Chapter 5.****Oxidative Stress in the Aging Rat Heart is Reversed by Dietary Supplementation  
with (*R*)- $\alpha$ -Lipoic Acid**

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## 5.1 Abstract

Oxidative stress has been implicated as a causal factor in the aging process of the heart and other tissues. To determine the extent of age-related myocardial oxidative stress, oxidant production, antioxidant status and oxidative DNA damage were measured in hearts of young (2 months) and old (28 months) male, Fischer 344 rats. Cardiac myocytes isolated from old rats showed a nearly 3-fold increase in the rate of oxidant production compared to young rats, as measured by the rates of 2,7-dichlorofluorescein diacetate oxidation. Determination of myocardial antioxidant status revealed a significant 2-fold decline in the levels of ascorbic acid ( $p=0.03$ ), but not  $\alpha$ -tocopherol. In addition, a significant age-related increase ( $p=0.05$ ) in steady-state levels of oxidative DNA damage was observed, as monitored by 8-oxo-2'-deoxyguanosine levels. To investigate whether dietary supplementation with (R)- $\alpha$ -lipoic acid (LA) was effective at reducing oxidative stress, young and old rats were fed an AIN-93M diet with or without 0.2% (w/w) LA for 2 weeks prior to sacrifice. Cardiac myocytes from old, LA supplemented rats exhibited a markedly lower rate of oxidant production, which was no longer significantly different from that in cells from unsupplemented, young rats. Lipoic acid supplementation also restored myocardial ascorbic acid levels and reduced oxidative DNA damage. Our data indicate that the aging rat heart is under increased mitochondria-induced oxidative stress, which is significantly attenuated by lipoic acid supplementation.

## 5.2. Introduction

Aging is associated with an increased incidence of cardiac arrhythmias and diastolic and systolic dysfunction, which may ultimately lead to heart failure. Heart failure alone is the leading cause of hospitalization, permanent disability and death in persons over the age of 65 [129] in the United States. Because of the enormous suffering and health-care burden that cardiac dysfunction causes, much effort has gone into understanding the mechanisms leading to age-related myocardial decline. It has been difficult, however, to separate the effects of aging per se from those of age-associated diseases (atherosclerosis, diabetes, hypertension) on cardiac performance. Thus, the relative contribution of “aging” to myocardial dysfunction is not well defined.

Even though the mechanisms leading to alterations in cardiac performance are not well understood, there is reason to suspect increased oxidative stress to significantly contribute to myocardial dysfunction with age. It is generally agreed that isolated mitochondrial preparations from old compared to young hearts produce more reactive oxygen species (ROS), reflecting an age-related decline in coupling of electron transport to ATP production. These changes to mitochondria may lead to the reported increase in superoxide and hydrogen peroxide production in mitochondria prepared from old versus young rats [16,130,131]. Thus, it is conceivable that dietary interventions with antioxidants, which could augment endogenous antioxidant compounds to either prevent the formation or quench the higher levels of oxidants,

could provide an effective means to improve or maintain myocardial function with age.

(*R*)- $\alpha$ -lipoic acid (LA) is a thiol compound found naturally in plants and animals [42]. Lipoamide dehydrogenases, found only in mitochondria, reduce free LA to dihydrolipoic acid (DHLLA), which is a potent antioxidant. Thus, LA supplementation may increase cellular and mitochondrial antioxidant status, thereby effectively attenuating any putative increase in oxidative stress with age [43].

Aside from acting as a potent antioxidant in its own right, LA increases or maintains levels of other low molecular weight antioxidants, particularly glutathione (GSH) and ascorbic acid (AA). LA may exert these effects by “sparing” both GSH and vitamin C [44] or, in the case of GSH, by increasing the cellular uptake of cysteine [45], which is the rate limiting substrate for GSH biosynthesis.

The purpose of the present study was two-fold: 1) to examine the age-related changes to myocardial oxidant production, low molecular weight antioxidant status and indices of oxidative damage, and 2) to determine whether dietary supplementation of lipoic acid could improve those indices of oxidative stress. Overall, our results show that the aging rat myocardium exhibits increased oxidant production, significantly lower ascorbic acid levels and a marked increase in the steady-state levels of oxidative DNA damage. LA supplementation significantly reverses the age-related decline in myocardial ascorbic acid content, and lowers the rate of oxidant production and the steady-state levels of oxidative DNA damage. Our results thus

indicate that dietary supplementation with lipoic acid may be an effective means to lower increased myocardial oxidative stress with age.

### 5.3 Materials and Methods

#### *Materials*

The following chemicals were used: EGTA (ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid), heparin (sodium salt), Rhodamine 123, dithiothreitol, L-ascorbic acid, meta-phosphoric acid, and 8-oxo-dG standard (Sigma, St. Louis, MO); 2',7'-dichlorofluorescein diacetate (DCFH) (Molecular Probes, Eugene, OR); collagenase, type 2 (Worthington, Lakewood, NJ); nuclease P1, alkaline phosphatase, sodium iodide, proteinase K, and RNAase A (Roche Pharmaceuticals, Indianapolis, IN). All other reagents were reagent grade or better.

#### *Animals*

Rats (Fischer 344, virgin male, outbred albino), both young (2 to 5 months) and old (24 to 28 months; National Institute of Aging animal colonies), were acclimatized in the Oregon State University animal facilities for at least 1 week prior to experimentation. Animals were placed on an AIN-93M standard diet; some rats were given AIN-93M diet supplemented with 0.2% (w/w) (*R*)- $\alpha$ -lipoic acid (Asta Medica, Germany) for two weeks prior to sacrifice. Water was given *ad libitum* throughout.

In experiments examining tissue antioxidant and oxidative DNA damage levels, rats were anesthetized with diethyl ether and a midline incision was made in the abdomen. Animals were sacrificed by cutting through the diaphragm, followed by severing the superior vena cava. Hearts were quickly removed, cut into small pieces, and the pieces placed individually in cryotubes and snap frozen in liquid nitrogen.

### *Cardiac myocyte isolation.*

For analysis of cellular oxygen consumption, average mitochondrial membrane potential and oxidant production, the heart was dispersed into single cells by perfusion with 1% collagenase [132]. Typically, the isolation procedure yielded  $5.0\text{--}7.5 \times 10^6$  calcium-tolerant ventricular cells per heart that exhibited typical rod-shaped appearance and morphological striations. Cell viability, as measured by Trypan Blue exclusion, was typically between 60 to 80%. These values are in agreement with those cited in the literature [133].

It was necessary to modify the above procedure in order to isolate cells from old rats. Old rats had to be injected with twice the amount of heparin to remove all blood from the heart. The amount of collagenase perfused through the heart was also increased to 1.2% (w/v) and the perfusion flowrate was raised from 2.8 to 7 ml/min. These changes were necessary because of the increased fibrotic nature of the heart in old compared to young rats. Even with these modifications cell yield and viability were more variable than that for young rats:  $3.0\text{--}7.0 \times 10^6$  cells isolated per heart from old rats with viability ranging from 45 to 80%. Because of the possibility that low cell viability and yield would result in data that do not accurately represent the *in vivo* situation, no isolated cell preparations were used that had an initial viability below 70% and a yield of  $<5.0 \times 10^6$  cells per heart.

### *DCFH measurement*

Formation of oxidants in isolated cardiac myocytes was determined by assaying the fluorescence of 2',7'-dichlorofluorescein (DCF), the oxidation product of 2',7'-dichlorofluorescein (DCFH). Duplicate samples were routinely monitored. Fluorescence was measured using a Hitachi F-2500 fluorescent spectrophotometer

(Hitachi Instruments, Tokyo, Japan) using standard fluorescein filters and Hitachi-supplied software. To determine whether age-related changes to cellular oxygen consumption affected apparent changes in oxidant production, cellular oxygen consumption was measured and data expressed as the fluorescence change per  $\mu\text{mol O}_2$  consumed/ $10^6$  cells.

#### *Ascorbic acid analysis*

Ascorbate was measured essentially as in [62]. Briefly, tissue homogenates were mixed with an equal volume of *meta*-phosphoric acid (10% w/v) containing 1 mmol/l of the metal chelator diethylenetriaminepentaacetic acid (DTPA) and centrifuged to remove the precipitated proteins. Ascorbic acid was separated by HPLC [62] and detected at an applied potential of +0.6 V by a LC 4B (Bioanalytical Systems Inc., West Lafayette, Ind.) electrochemical detector.

#### *Vitamin E analysis*

Myocardial vitamin E levels were determined as in [134]. Briefly, myocardial tissue (50 mg) was homogenized in 10 mM phosphate buffered saline containing 1 mmoles DTPA. 50  $\mu\text{l}$  of the homogenate was extracted in hexane:methanol (5:1) and the hexane phase was collected and dried under a constant stream of nitrogen. The sample was reconstituted with methanol and analyzed by HPLC with electrochemical detection at an applied potential of +0.5 V by a LC 4B (Bioanalytical Systems Inc., West Lafayette, Ind.) electrochemical detector.

#### *Oxidative DNA damage.*

Analysis of oxidative damage to nuclear DNA was measured by the method of Helbock et al [135]. DNA was extracted using the chaotropic sodium iodide method

by a DNA Extractor WB kit (Wako BioProducts, Richmond, VA). DNA hydrolyzates were analyzed by HPLC with electrochemical coulometric detection, as described [135].

#### *Oxidative Lipid damage.*

Analysis of oxidative damage to lipids was quantified by measuring esterified isoprostanes in lipids using a highly precise and accurate assay employing gas chromatography/mass spectrometry [136].

#### *Statistical Analysis*

Statistical significance was determined by the paired Student's *t* test, using statview statistical software. Results are expressed as the mean  $\pm$  SEM. A P-value of less than 0.05 was considered significant.

## 5.4 Results

To determine the effects of aging on the general metabolic rate, O<sub>2</sub> consumption in freshly isolated cardiac myocytes from young and old rats was determined. O<sub>2</sub> consumption in cells from young rats was 3.18±0.53 mmol O<sub>2</sub>/min per 10<sup>6</sup> cells. In contrast, O<sub>2</sub> consumption in cardiac myocytes from old rats was 1.74±0.13 mmol O<sub>2</sub>/min per 10<sup>6</sup> cells, a 45% decline compared to young rats (Figure 5.1). These results suggest an age-associated decrease in the basal metabolic rate of the cardiac myocytes.

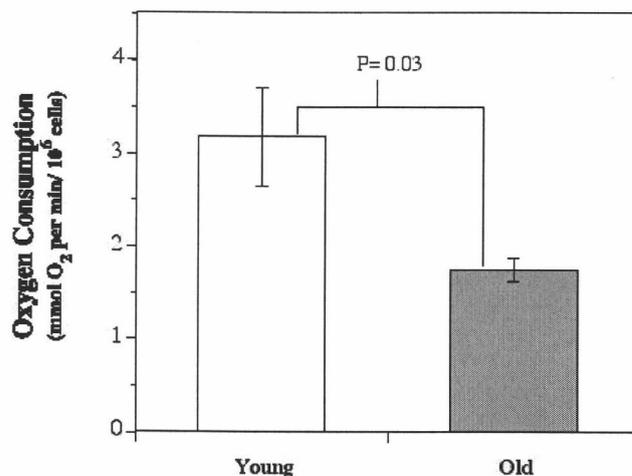
To examine how the decline in mitochondrial function affected cellular oxidant production, the rates of DCFH oxidation in freshly isolated cardiac myocytes from old and young rats were determined. In cardiac myocytes isolated from old rats, total cellular oxidant production was 125% higher than in myocytes from young rats (Figure 5.2). This increase in oxidant production was even more pronounced when normalized to the rates of consumption. Thus, the rate of DCFH fluorescence per mmole O<sub>2</sub> consumed was 3-fold higher in cardiac myocytes from old rats compared to young rats. These results strongly suggest that, with age, cardiac myocytes are under increased oxidative stress, possibly due to a decline in mitochondrial function.

To investigate the effects of this age-associated increase in oxidative stress on cellular antioxidant capacities, ascorbate and α-tocopherol levels in the freshly isolated hearts from old and young rats were measured. Consistent with the data on oxidant production (Figure 5.2), we observed a 57% decrease in the tissue ascorbate

concentration of old rats ( $1.4 \pm 0.87$  nmoles/mg protein) compared to young rats ( $3.08 \pm 1.1$  pmoles/mg tissue ( $p = 0.02$ ); Table 5.1). In contrast, no age-related decrease in vitamin E levels was observed ( $0.97 \pm 0.29$  and  $1.16 \pm 0.62$  nmoles/mg protein in hearts from young and old). This result suggests that there is a differential consumption of water-soluble versus lipid-soluble antioxidants in vivo due to aging.

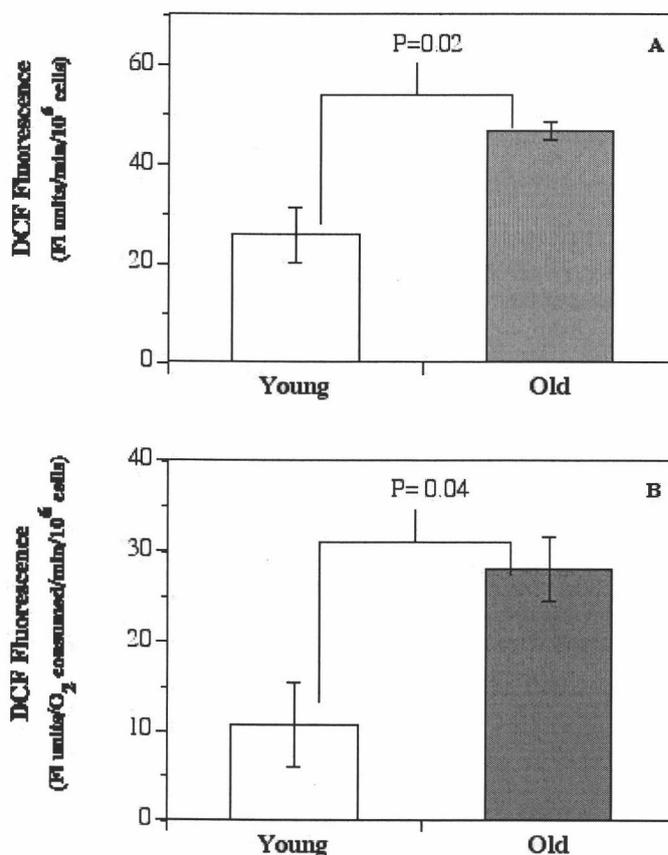
To further explore this notion of different levels of oxidative stress with aging in the aqueous and lipid compartments, respectively, we examined oxidative damage to DNA, as assessed by the levels of 8-oxo-dG, and lipids, as assessed by levels of F<sub>2</sub>-isoprostanes. Consistent with the pattern of antioxidant depletion with age, we found a two-fold increase in oxidative DNA damage in the heart of old rats ( $4 \pm 0.4$  8-oxo-dG/105 dG bases) compared to young rats ( $2.01 \pm 0.25$  8-oxo-dG/105 dG bases ( $p = 0.002$ ); Fig. 4). In contrast, the levels of F<sub>2</sub>-isoprostanes in hearts from old rats ( $3.74 \pm 0.41$  ng/g wet tissue) were not significantly different from those in young rats ( $3.47 \pm 0.017$  ng/g wet tissue). These results suggest that within the heart, macromolecules in an aqueous environment may be more susceptible to damage due to age-associated increase in oxidative stress than membrane lipids.

In light of the above evidence of increased oxidative stress in the aging heart, we examined whether supplementation with (*R*)- $\alpha$ -lipoic acid can reverse the decline in mitochondrial function and reduce oxidative stress. To this end, Fischer 344 rats of varying ages ((young (2-4 mo) and old (24-28 mo)) were fed an AIN-93M diet with or without 0.2% (w/w) (*R*)- $\alpha$ -lipoic acid for two weeks. Results show a significant decrease in the cellular oxidant production in the old LA-supplemented versus old



**Figure 5.1. Age-related O<sub>2</sub> consumption in isolated cardiac myocytes declines.**

O<sub>2</sub> consumption in isolated cardiac myocytes from young and old rats was monitored using an oxygen electrode. Results show that O<sub>2</sub> consumption, an indicator of cellular metabolic rate, declines sharply with age. Results are expressed as mean  $\pm$  SEM for cells from young rats (N=5) and the mean  $\pm$  SEM (N=5) for cells from old rats.



**Figure 5.2 Cardiac myocytes produce significantly more oxidants with age.**

A. Cells from old rats (24 mo) produce ~45% more oxidants, as measured by DCFH fluorescence, than cells from young (3 mo) rats. B. The rate of oxidant production is even more marked when oxidant production is normalized to the cellular rate of O<sub>2</sub> consumption. Results are expressed as the mean  $\pm$  SEM of 3 experiments.

**Table 5.1. Age associated changes in antioxidant levels and oxidative damage in the heart.**

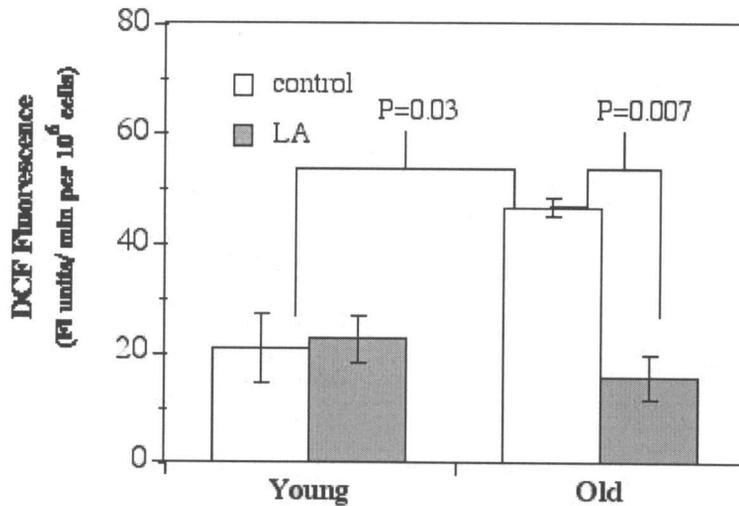
Age Group	Young	Old
<b>Ascorbate</b> (nmoles/mg protein $\pm$ SEM)	3.08 $\pm$ 1.1	1.4 $\pm$ 0.87*
<b><math>\alpha</math>-Tocopherol</b> (nmoles/mg protein $\pm$ SEM)	0.97 $\pm$ 0.29	1.16 $\pm$ 0.62
<b>8-oxo-dG</b> (# 8-Oxo-dG/1x10 <sup>5</sup> dG bases)	2.01 $\pm$ 0.25	4 $\pm$ 0.4#
<b>F<sub>2</sub>-isoprostanes</b> (ng/g wet tissue)	3.47 $\pm$ 0.017	3.74 $\pm$ 0.41

\* P = 0.02 ; # P=0.002

Ascorbate and  $\alpha$ -tocopherol levels in the freshly isolated heart from young and old rats were measured using electrochemical detection following separation by HPLC. The results show a significant decline in the ascorbate levels. In contrast, lipid soluble antioxidant,  $\alpha$ -tocopherol did not change with age. As a measure of oxidative damage to DNA and lipid, 8-oxo-dG and F<sub>2</sub>-isoprostanes were measured by using coulometric electrochemical detection and GC-MS. The results show a two-fold increase in steady state levels of 8-oxo-dG. However, there was no change in the steady state levels of F<sub>2</sub>-isoprotanes with age. The results are expressed as mean  $\pm$  SEM and statistical analysis was done by using student t-test.

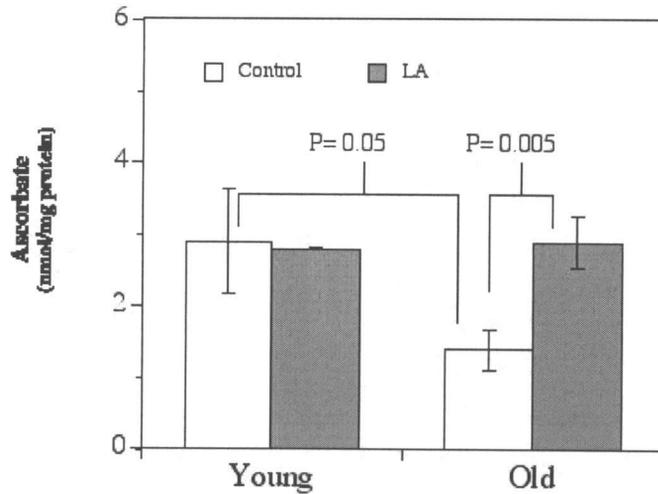
unsupplemented animals (Figure 5.3). In agreement with these data, we also found a significant two-fold improvement in ascorbate levels in the hearts from old LA-treated versus untreated animals ( $2.9 \pm 0.36$  versus  $1.4 \pm 2.9$  nmoles/mg protein, respectively;  $p=0.03$ ). In fact, cardiac ascorbate levels in LA-fed old rats were not different from those in young rats ( $2.9 + 0.717$  nmoles/mg protein; Figure 5.4). However, a similar increase in tissue ascorbate levels was not achieved by LA-feeding to young rats. Furthermore, there was no effect of LA treatment on the levels of  $\alpha$ -tocopherol.

To investigate whether the decreased ROS production and increased ascorbate levels in LA-treated old rats translated into a decrease in oxidative damage to DNA and lipids, 8-oxo-dG and F2-isoprostane levels were measured. Indeed, we found a 30% decrease in cardiac 8-oxo-dG levels in the supplemented old rats ( $2.84 \pm 0.39$  8-oxo-dG/ $10^5$  dG bases) compared to their age-matched controls ( $4.00 \pm 0.40$  8-oxo-dG/ $10^5$  dG bases ( $p = 0.05$ ); Figure 5.5). In the young animals, LA supplementation did not alter the steady state levels of 8-oxo-dG in the heart. As expected, treatment with LA did not have any effect on the steady state levels of F2-isoprostanes both in young and old rats. Thus, by decreasing the rate of oxidant production and increasing the level of antioxidant protection in the aqueous phase, LA treatment caused a marked reversal in the steady state levels of 8-oxo-dG in old rats to the levels found in young rats.



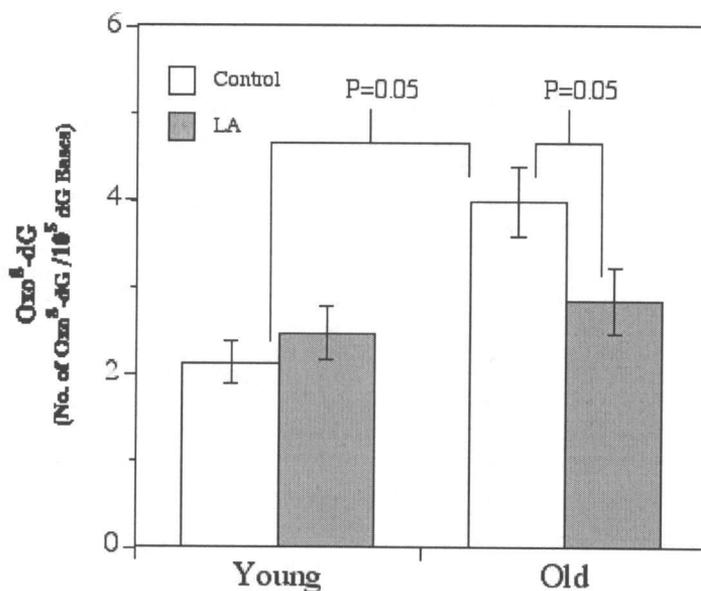
**Figure 5.3. A two-week regimen of LA markedly lowers the age-related increase in cellular oxidant production.**

Myocardial oxidant production in both young (2-4 mo; n= 9) and old (24-28 mo; n=8) treated with or without 0.2% (w/w) lipoic acid was measured. Results show that lipoic acid treatment significantly reversed the age-related increase in oxidant production back to levels that were not different from the young. Results are expressed as mean  $\pm$  SEM for heart isolated from young (n= 4), young LA (n=5), Old (n=4) and Old LA (n=4). Analysis of variance test was used for statistical analysis with the level of significance defined as  $p < 0.05$ .



**Figure 5.4. Dietary supplementation of LA leads to restoration of ascorbate back to the levels found in the young.**

Ascorbate levels in freshly isolated hearts from old and young animals were determined following two week supplementation of AIN-93M diet with or without 0.2% (w/w) (*R*)- $\alpha$ -lipoic acid. The ascorbate levels in the old supplemented animals (n=12) was significantly higher (p=0.03) than old unsupplemented animals (n=10) to the level no longer significantly different from young unsupplemented animals. Results are expressed as mean  $\pm$  SEM for heart isolated from young (3), young LA (n=3), Old (n=10) and Old LA (n=12). Analysis of variance test was used for statistical analysis with the level of significance defined as p < 0.05.



**Figure 5.5** LA supplementation leads to marked reduction in the steady state levels of 8-oxo-dG.

The steady state levels of 8-oxo-dG in freshly isolated hearts from old and young rats following a two-week supplementation with AIN-93M diet with or without 0.2% (w/w) (R)- $\alpha$ -lipoic acid by using coulometric detection following separation with HPLC. The results show that LA supplementation significantly lowered the age-related accumulation of 8-oxo-dG in hearts from old rats. Results are expressed as mean  $\pm$  SEM for heart isolated from young (n=8), young LA (n=3), Old (n=10) and Old LA (n=9). Analysis of variance test was used for statistical analysis with the level of significance defined as  $p < 0.05$ .

## 5.5 Discussion

Previous studies examining the age-related changes in mitochondria isolated from old animals have reported that mitochondrial oxidant production increases with age [16]. However, a recent study by Hansford and co-workers suggested that the increase in mitochondrial oxidant production observed with age may be attributed to an artifact stemming from assay conditions due to excessively high levels of substrate (succinate) used [17]. In addition, Hagen and co-workers have found that selective loss of defective mitochondria during the isolation process can further complicate the interpretation of results from studies using isolated mitochondria [137]. However, in this paper, we have avoided these potential problems by examining the age-associated changes in metabolic function and antioxidant status in intact cardiac myocytes isolated from young and old animals.

In the present study, we observed a significant increase in DCF fluorescence in myocytes from old when compared to young animals. This increase in oxidant levels may be attributed to at least three possibilities: 1) increased oxidant production mainly from decaying mitochondria, 2) decline in cellular antioxidant status with no increase in oxidant flux, and 3) both an increase in oxidant production and a decline in antioxidant status. The present work cannot distinguish between these possibilities. However, it is notable that we observed a significant decline in myocardial ascorbate levels and oxygen consumption with age. Lower oxygen consumption would seemingly be a compensatory mechanism to also lower mitochondrial oxidant flux.

Along with loss of ascorbate, our results would suggest that the decline in antioxidant status may be a significant contributing factor in the apparent age-related increase in myocardial oxidative stress.

In contrast to the age-related decrease in the levels of ascorbate and an increase in the levels of 8-oxo-dG, the levels of  $\alpha$ -tocopherol and F<sub>2</sub>-isoprostanes did not change with age. Because these moieties are primarily located in lipophilic environments, this data suggests that the effects of endogenous oxidants may be confined to the aqueous milieu of the heart. In support of this notion, we have previously found that F<sub>2</sub>-isoprostanes formation is reduced by vitamin E [138]. Ascorbate however, does not affect the formation of F<sub>2</sub>-isoprostanes, at least *in vitro* in microsomes [139].

One factor contributing to such compartmentalization may be due to the presence of redox-active transition metal ions in the cytosol which can catalyze the conversion of hydrogen peroxide and superoxide into the more highly reactive hydroxyl radicals via Fenton chemistry. Interestingly, previous *in vitro* experiments by Lodge and co-workers show that dihydrolipoic acid can inhibit copper induced LDL oxidation by direction chelation of free copper ions [55]. In addition, LA appears to inhibit iron-induced ascorbate oxidation by reducing redox-active iron. We are currently examining the nature of the interaction between transition metals and LA. Thus, in addition to its antioxidant effect, dihydrolipoic acid may protect against lipid peroxidation by chelating free metal ions *in vivo*.

Ascorbate may be consumed more rapidly than  $\alpha$ -tocopherol in vivo. First, the reduction potential of ascorbate (280 mV) is much lower than that of  $\alpha$ -tocopherol (500 mV) [36]. Thus, ascorbate is a more effective antioxidant capable of inhibiting lipid peroxidation against a number of different oxidant species [62]. Its low reduction potential also allows it to regenerate  $\alpha$ -tocopherol while the reverse is not possible [36]. Second, the concentration of ascorbate is 2 to 3 fold higher than  $\alpha$ -tocopherol; thus, ascorbate may spare  $\alpha$ -tocopherol from oxidation.

In contrast to a previous study showing an increase in urinary  $F_2$ -isoprostanes with age, our results show that myocardial levels of  $F_2$ -isoprostanes did not increase [140]. Myocardial  $F_2$ -isoprostanes may not accumulate due to rapid repair and/or clearance, whereas plasma or urinary  $F_2$ -isoprostanes would be reflective of released  $F_2$ -isoprostanes from all tissues and renal clearance.

The beneficial effects of dietary supplementation of (*R*)- $\alpha$ -lipoic acid towards ameliorating the age-related changes in cardiac myocytes may be attributed to its ability to act as an antioxidant as well as its role as in modulating metabolism. Along with the reduction in oxidant production, (*R*)- $\alpha$ -lipoic acid supplementation restores myocardial ascorbate levels from old rats. Previous work by Lykkesfeldt and co-workers have reported that there is no change in hepatic ascorbate synthesis with age [141], which suggests that (*R*)-lipoic acid may restore myocardial ascorbate levels by stimulating hexose transporter activity and/or by direct regeneration of dehydroascorbate. Furthermore, LA can indirectly increase GSH levels by increasing

cystein uptake, which is a rate-limiting step for GSH biosynthesis. This increase in thiol antioxidant levels can in turn enhance the rate of ascorbate recycling.

Dietary supplementation of LA also leads to significantly lower steady state levels of 8-oxo-dG in hearts from old animals. This reduction suggests that the age-related accumulation of 8-oxo-dG *in vivo* may be due to increased oxidative insult rather than decreased repair capacity. In support of this, a recent study by Souza-Pinto and co-workers has reported that there is an age-dependent increase in 8-oxo-dG glycosylase/AP lyase activity in rat mitochondria with age [142].

The causes for this age-related decline in myocardial mitochondrial functions are not completely understood. One possibility is that with age, there may be a loss of essential cofactors such as LA, which may limit optimal mitochondrial performance. Supplementation with LA may replenish needed cofactor for  $\alpha$ -keto acid dehydrogenases (pyruvate oxidoreductase and 2-oxo-glutarate oxidoreductase) used in pyruvate and fatty acid metabolism. It has been shown that the proportion of the active form of pyruvate oxidoreductase declines with age [143], possibly due to modification of the lipoamide moiety of the E2 subunit [144]. Thus, feeding old rats LA may reverse the age-associated decline in LA-dependent oxidoreductase activity. This may also explain why we only observed a beneficial effect of LA only in old and not in young animals.

Other necessary cofactors required for mitochondrial function, such as carnitine and cardiolipin, also decline with age [145,146]. Carnitine loss, for example, can limit the transport of fatty acids to mitochondria for  $\beta$ -oxidation, which is the

major source for ATP synthesis. In addition, decline in cardiolipin has been shown to decrease substrate transport in isolated mitochondrial preparations and lower cytochrome *c* oxidase activity [146].

One possible physiological consequence of these decreased cofactors would be loss of ATP production, which may lead to cardiac stiffness. To maintain myocardial function, a constant supply of ATP is required and little reserves are maintained. This suggests that when energy supply is interrupted (ischemia) or impaired (aging), ATP levels decline rapidly. Like systolic contraction, diastolic relaxation also requires high levels of ATP, because ATP acts as an allosteric effector to disassociate actin from myosin [147]. Thus, any decrement in mitochondrial ATP synthesis affects cardiac stiffness appreciably. A decline in ATP synthesis also compromises  $\text{Ca}^{+2}$  re-uptake into the sarcoplasmic reticulum from the cytosol, again affecting myocardial relaxation [148,149]. The  $\text{Na}^{+}/\text{Ca}^{+2}$  transporter is also energy-dependent and a decline in myocardial ATP levels would thus slow cardiac relaxation by decreasing the rate of  $\text{Ca}^{+2}$  removal from the cytosol [148,149]. It is notable that a general attribute of myocardial aging is a prolonged cytosolic calcium transient and slower myocardial relaxation rate [150,151].

The exact physiological consequences associated with these cellular changes remains to be elucidated. Although these changes may not affect the hearts function under normal conditions, it is possible that a loss in bio-energetic capacity along with antioxidant protection may severely limit the hearts ability to respond to physical

stress. More studies are needed to carefully correlate the limitation brought about by age-associated changes in the mitochondrial function.

In conclusion, our present findings would suggest that (*R*)- $\alpha$ -lipoic acid supplementation may be a safe and effective means of improving systemic decline in over all metabolic function and also increase protection against both endogenous and external production of ROS. However, long-term feeding studies with LA are needed to determine whether benefits of LA seen in old animals can be sustained over time.

## **5.6 Acknowledgement**

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**Chapter 6.**

**Two subpopulations of mitochondria in the aging rat heart display heterogeneous levels of oxidative stress**

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## 6.1 Abstract

Cardiac mitochondria are composed of two distinct subpopulations: one beneath the sarcolemma (subsarcolemmal mitochondria: SSM), and another along the myofilaments (interfibrillary mitochondria: IFM). Previous studies suggest a preferential loss of IFM function with age; however, the age-related changes in oxidative stress in these mitochondrial subpopulations have not been examined. To this end, the changes in mitochondrial antioxidant capacity, oxidant output and oxidative damage to Complex IV in IFM and SSM from young and old rats were studied. Results show no apparent differences in any parameters examined between IFM and SSM from young rats. However, relative to young, only IFM from *old* rats had a significantly higher rate of oxidant production and a decline in mitochondrial ascorbate levels and GSH redox status. The age-related decline in mitochondrial antioxidant capacity in IFM was accompanied by a marked loss in glutaredoxin and GSSG reductase activities, suggesting a diminished reductive capacity in IFM with age. Moreover, the loss in Complex IV activity was limited to the IFM of old rats, which was accompanied by a four-fold increase in 4-hydroxynonenal-modified Complex IV. Thus, mitochondrial decay is not uniform and further indicates that myofibrils may be uniquely under oxidative stress in the aging heart.

## 6.2 Introduction

The aging heart undergoes significant functional and structural alterations leading to atrophy and a compensatory hypertrophy, followed by myocardial fibrosis [152,153]. In addition, there is an age-related decline in the capacity to withstand stress, such as ischemia/reperfusion [154-156]. In its most severe form, cardiac decay results in congestive heart failure, one of the leading causes of death in people over the age of 65. Although mechanisms underlying cardiac decay are not clear, loss of mitochondrial function and a resultant increase in oxidative stress has been proposed to be one of the key factors in myocardial aging [131,157,158].

The extent of age-related changes to cardiac mitochondria are poorly defined and further confounded by their heterogeneous nature. Similar to other muscle tissues, cardiac mitochondria are distributed into two localized groups. Subsarcolemmal mitochondria (SSM) are adjacent to the subsarcolemmal membrane while interfibrillary mitochondria (IFM) are tightly associated with myosin fibers [18]. In addition to differences in their locality, the rates of oxidative phosphorylation of IFM and SSM are also different. In comparison to SSM, IFM exhibit higher State 3 respiratory rates and contain increased content of respiratory cytochromes and activity of electron transport chain complexes. Due to the differences in intracellular locale, SSM may be more important in supplying ATP for ion homeostasis (Ca-ATPases), while IFM may supply ATP required for myosin ATPase activity. Understanding the

age-related changes specific to these subpopulations will be fundamentally important in identifying likely cellular processes that are affected during the aging process.

Interestingly, Hoppel and co-workers reported that the age-related loss in respiratory function is primarily localized to IFM [159]. This is reflected by the fact that aging results in a selective loss of IFM protein yield [159] and activities of Complexes III [160] and IV [159]. The aging defects on Complex III appear to involve lesions at the cytochrome c binding site, while the exact nature of defects involving Complex IV have not been established. The tight physical association of IFM to myosin fibrils necessitates the use of proteases during the isolation procedure and thus may be more prone to damage during isolation. However, Hoppel and co-workers demonstrated that SSM treated in an identical manner, as IFM exhibit no changes in bioenergetic parameters, such as respiratory control ratios, and in various electron transport chain activities [161].

The selective decline in IFM function raises an important issue as to whether aging results in a disproportionate increase in oxidative stress in IFM versus SSM. However, to date no information is available on the extent to which aging affects various parameters of oxidative stress in these two subpopulations of cardiac mitochondria. The redox environment of mitochondria is controlled, in part, by reductants, such as NAD(P)H, ascorbate and glutathione (GSH). In addition, GSH and thioredoxin-dependent enzymes play a major role in maintaining reducing equivalents required for enzyme function and signal transduction [162,163]. An age-related decline in any of these systems would likely result in an enhanced pro-oxidant

environment and subsequent oxidative injury to critical mitochondrial proteins. Theoretically, this may provide some explanation as to the selective decline in IFM function. In this report, we show that IFM are prone to oxidative stress and injury, which is in part due to a selective loss in thiol antioxidant systems in this particular mitochondrial subpopulation.

### 6.3 Materials and Methods

#### *Animals*

Rats (male Fischer 344), both young (2 to 5 months: N=5) and old (24 to 28 months; N=8; National Institute of Aging animal colonies), were acclimatized in the Oregon State University animal facilities for at least 1 week prior to experimentation. Animals were maintained on standard chow diet and water was given ad libitum. Rats were anesthetized with diethyl ether and a midline incision was made in the abdomen. Heparin (0.4 mg/ml) was injected via the femoral vein and Hank's balanced salt buffer (pH 7.4) was perfused through the superior vena cava for 5 min to remove blood. Hearts were quickly removed for mitochondrial isolation.

#### *Mitochondrial isolation*

Two subpopulations of cardiac mitochondria were isolated according to the procedures of Palmer *et al* [18]. Briefly, hearts were rinsed and finely minced in buffer A (220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L 3-[N-Morpholino]propanesulfonic acid (MOPS), pH 7.4) containing 2 mM ethylene glycol-bis( $\beta$ -aminoethyl ether) tetraacetic acid (EGTA), 0.2% bovine serum albumin (BSA). The minced tissue was homogenized with a teflon-glass homogenizer and centrifuged at 500 x g at 4 °C. The resulting pellet was re-suspended to its original volume in buffer A and homogenized. The supernatants from these preparations were pooled and centrifuged at 3000 x g at 4 °C for 10 min to obtain the subsarcolemmal mitochondrial

fraction. Isolated SSM was washed twice in buffer A containing 0.5 mM EGTA and kept on ice until used. The remaining tissue pellet obtained during SSM isolation was resuspended in buffer B containing 100 mmol/L KCl, 2 mmol/L EGTA, 0.2% BSA 50 mmol/L MOPS, pH 7.4. To these samples, Nagarse (Sigma) was added to a final concentration of 5 mg/g wet weight of tissue and homogenized immediately with a dounce-type tissue homogenizer. This homogenate was diluted two-fold with buffer B and centrifuged at 5000 x g for 5 min. The remaining pellet following Nagarse treatment was resuspended with buffer B and sedimented at low speed to obtain the nuclear fraction. The pellet was washed twice in buffer B to ensure maximal yield. The resulting supernatants were pooled and centrifuged at 3000 x g at 4 C for 10 min. Interfibrillary mitochondria (IFM) were washed twice and resuspended in buffer B (without BSA). The protein yield was quantified using standard Lowry assay with BSA as the external standard.

#### *Mitochondrial oxidant generation*

The rate of 2',7'-dihydrodichlorofluorescein (DCFH) oxidation was quantified to determine changes in mitochondrial oxidant generation [14]. Mitochondria (25  $\mu$ g/ml) were incubated in the presence of respiration buffer [14] containing 0.1 mM ATP, 2 mM ADP, 50  $\mu$ M malate and glutamate in 96 well plates. DCFH (25  $\mu$ mol/L) was added and the change in DCFH oxidation was monitored for 40 min at 30 C using a cytoflour 4000 fluorescence plate reader (Applied Biosystems). In order to minimize the potential enhancement of oxidant generation due to the presence of high substrate

concentrations [17], glutamate and malate ( $50 \mu\text{M}$ ) were used. The low levels of substrate were not limiting since the rate of oxidant generation in the presence of KCN was maintained at a level which was at least 10-fold higher than that of normal, tightly coupled mitochondria (data not shown).

#### *Ascorbate and GSH analysis*

Mitochondria ( $750 \mu\text{g}$  protein) were used for the detection of ascorbate and GSH. Ascorbate was measured according to the method of Frei *et al* [62]. Ascorbate was separated by HPLC [62] and detected at an applied potential of  $+0.6 \text{ V}$  by a LC 4B electrochemical detector (Bioanalytical Systems, Inc).

Mitochondrial GSH status was measured according to the method of Jones *et al* [124]. GSH and GSSG were separated by HPLC and detected using a fluorescence spectrophotometer (Hitachi Fl-6000), with monochromators set at  $335 \text{ nm}$  for excitation and  $515 \text{ nm}$  for emission.

#### *Mitochondrial antioxidant enzyme assays*

Mitochondrial thioredoxin reductase activity was determined based on the methods of Hill and co-workers [164]. Mitochondria ( $10 - 40 \mu\text{g}$  protein) were incubated in potassium phosphate buffer ( $100 \text{ mmol/L}$ ;  $\text{pH } 7.0$ ) in the presence of  $5 \text{ mmol/L}$  5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB),  $20 \text{ mmol/L}$  EDTA, and  $0.2 \text{ mg/ml}$  BSA for  $5 \text{ min}$ . Following the initial incubation,  $0.2 \text{ mmol/L}$  NADPH was added in the presence or absence  $20 \mu\text{mol/L}$  auranofin (a specific inhibitor of

thioredoxin reductase). Thioredoxin reductase activity was obtained based on the auranofin inhabitable rate of DTNB reduction.

Mitochondrial GSSG reductase activity was measured according to the method of Smith and coworkers [165]. Briefly, mitochondria (40-100  $\mu\text{g}$  protein) were incubated in potassium phosphate buffer (100 mmol/L: pH 7.5) containing 0.2 mmol/L GSSG. To these samples, NADPH (0.2 mmol/L) was added and the rate of NADPH loss was monitored by following the absorbance loss at 340 nm.

Mitochondrial glutaredoxin reductase activity was determined according to the method of Gladyshev and co-workers [166]. Mitochondria (20- 50  $\mu\text{g}$  of protein) were added to a potassium phosphate buffer (100 mmol/L: pH 7.5) containing 0.2 mmol/L NADPH, 0.5 mmol/L GSH, and 0.4 units of GSSG reductase. To these samples, hydroxyethylidisulfide (HEDS: 2 mmol/L) was added and the decrease in absorbance of NADPH at 340 nm was monitored for 10 min. One unit of enzyme activity was defined as one  $\mu\text{mol}$  of NADPH oxidized per min.

#### *Complex IV (cytochrome c oxidase) Activity*

Cytochrome c oxidase activity of isolated mitochondria was measured by using a commercially available kit from Sigma (Cytoc-ox1). Mitochondria (2  $\mu\text{g}$  protein) were added to the assay buffer, which consisted of 10 mmol/L Tris-HCl, pH 7.0 and 120 mmol/L KCl. To these samples 50  $\mu\text{l}$  of reduced cytochrome C (0.22 mmol/L) were added and changes in absorbance at 550 nm were monitored for 1 min. An extinction coefficient of  $21,840 \text{ M}^{-1}\text{cm}^{-1}$  was used to quantify the activity.

### *Blue-Native Gel Electrophoresis*

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was carried out on a 5-13.5% linear acrylamide gradient according to the methods of Schagger *et al* [167]. Mitochondria (200  $\mu$ g of protein) were centrifuged at 10,000 x g for 10 min. The resulting pellet was reconstituted in 40  $\mu$ l of 50 mmol/L Bis-Tris (pH 7.0), containing 750 mmol/L 6-aminocaproic acid, and 5  $\mu$ l of n-dodecyl-B-D-maltoside (10% w/v). These samples were centrifuged at 100,000 x g for 15 min. Supernatants were collected and 5% (w/v) Coomassie blue brilliant G-250 (BioRad) dissolved in 1 M aminocaproic acid was added at a ratio of 1:14 (dye to sample volume ratio). Bovine liver catalase (Sigma, 230 kDA and 460 kDA) was used as a molecular weight marker.

### *Detection of 4-HNE adduction to mitochondrial electron transport chain proteins*

Electron transport chain complexes were separated using BN-PAGE and transferred onto PVDF membranes at a constant voltage of 50 V for 45 min. Immediately following the transfer, excess Coomassie dye was removed by a 30 min incubation at 50 °C in stripping buffer (100 mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.7). Membranes were probed with anti-4-hydroxynonenal (4-HNE) polyclonal rabbit sera (Advanced Life Technology). Following the detection with 4-HNE antisera, membranes were stripped and re-probed using monoclonal antibodies against Complex IV (Molecular Probes). Immunoreactive proteins were

detected using horseradish peroxidase conjugated secondary antibodies and were visualized by chemiluminescence detection (Amersham). Relative densities of the bands were digitally quantified using NIH image analysis software.

### *Statistical Analysis*

Results are shown as mean  $\pm$  standard errors for the indicated sample size. Statistical significance was assessed by single factor ANOVA with Tukey's post-hoc test. Differences were considered statistically significant at  $p < 0.05$ .

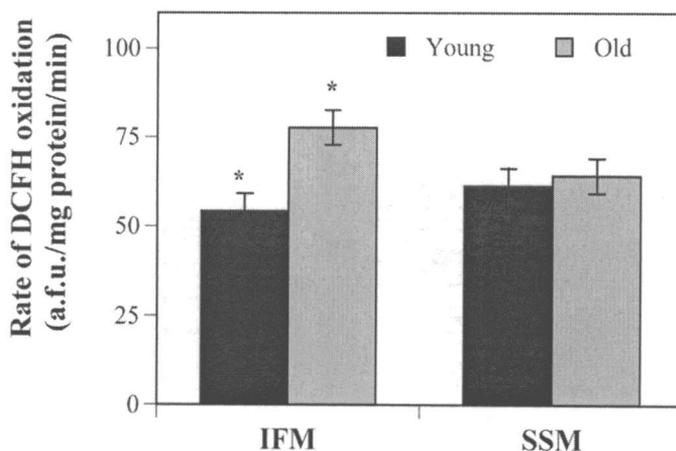
## 6.4 Results

### *Age-associated changes in mitochondrial oxidant generation*

The rate of DCFH oxidation was monitored to determine any age-associated differences in the rates of oxidant generation in SSM and IFM. As shown in Figure 6.1, no significant differences in oxidant production were observed between IFM ( $54.25 \pm 9.5$  a.f.u./mg protein/min) and SSM ( $61.4 \pm 5.4$  a.f.u./mg protein/min) isolated from young rats. Likewise, we observed no differences in the respiratory control and ADP/O ratios in IFM and SSM isolated from young animals (data not shown).

Comparing the rate of oxidant appearance in mitochondrial subpopulations from young and old rats showed that only the IFM were adversely affected with age. A significant 51% ( $p < 0.03$ ) increase in the rate of oxidant appearance was seen in IFM isolated from old ( $77.57 \pm 4.5$  a.f.u./mg protein/min) versus young rats ( $54.25 \pm 9.5$  a.f.u./mg protein/min; figure 6.1). The age-related increase in the rate of DCFH oxidation was not due to compromised mitochondrial integrity. The respiratory control ratios in old IFM ( $5.61 \pm 1.9$ ) were similar to that of young IFM ( $5.51 \pm 0.89$ ). However, the ADP/O ratios in old IFM ( $1.27 \pm 0.1$ ) were lower than young IFM ( $1.93 \pm 0.25$ ), suggesting that IFM from old rats utilize oxygen less efficiently relative to controls.

While IFM exhibit an increase in the rate of oxidant appearance with age, SSM did not show similar age-related changes in the rate of DCFH oxidation (Figure 6.1). In addition, the ADP/O and RCR in SSM remained unchanged with age (data not



**Figure 6.1 Age-related increase in mitochondrial oxidant generation is limited to IFM.**

The rate of mitochondrial oxidant generation under glutamate, malate, and ADP-stimulated conditions was examined as described in the materials and methods. Results show that the rate of mitochondrial oxidant appearance increases markedly in IFM isolated from old (open bar) when compared to young (closed bar). Results are expressed as mean  $\pm$  SEM for IFM and SSM isolated from young rats (N=5) and old rats (N=8).

(\* denotes significant ( $p < 0.05$ ) difference between identical mitochondrial preparations from young and old rats)

**Table 6.1 Age-associated changes in mitochondrial low molecular weight antioxidants**

	Young		Old	
	IFM	SSM	IFM	SSM
Ascorbate (nmol/mg protein)	1.22±0.13*	1.47±0.20	0.34±0.07* <sup>†</sup>	0.97±0.30 <sup>†</sup>
GSH (nmol/mg protein)	3.81±0.54*	3.75±0.26	2.32±0.29* <sup>†</sup>	3.58±0.47 <sup>†</sup>
GSSG (nmol/mg protein)	0.37±0.08*	0.17±0.09	0.54±0.1* <sup>†</sup>	0.12±0.08 <sup>†</sup>
Total GSH (nmol/mg protein)	4.69±0.57	4.11±0.34	3.38±0.2	3.82±0.52
% GSH Oxidized	16.3±2.9* <sup>†</sup>	8.2±3.6 <sup>†</sup>	32.18±6.88* <sup>†</sup>	6.2±1.15 <sup>†</sup>

Values are expressed as Mean±SEM

Ascorbate and GSH levels in the freshly isolated IFM and SSM from young and old rats were measured as described in the methods. The results show a significant decline in the ascorbate and reduced GSH levels. In contrast to ascorbate, total GSH levels did not change with age or type of mitochondria. The results are expressed as mean ± SEM. (\* denotes significant (p<0.05) difference between identical mitochondrial preparations from young and old rats while <sup>†</sup> denotes differences between IFM and SSM within the same age-group)

shown). These results suggest that IFM in the aging heart are solely responsible for increased oxidant generation previously observed [158].

*Age-associated changes in low-molecular weight antioxidant and thiol redox status*

To determine whether age-associated changes in low molecular weight antioxidants could, in part, account for the differences in oxidant generation displayed by IFM and SSM, mitochondrial ascorbate and glutathione (GSH) status were determined. Results show that IFM and SSM from young animals maintained similar levels of ascorbate and GSH (Table 6.1).

Despite the lack of differences in antioxidant concentrations between IFM and SSM in young rats, aging resulted in a selective loss of these antioxidants in IFM isolated from old animals. On an age-basis, the level of ascorbate present in old IFM was 73% lower ( $p < 0.002$ ) than in IFM obtained from young rats (Table 6.1). Similarly, IFM from old rats exhibited a 40% loss ( $p = 0.02$ ) in GSH levels when compared to IFM isolated from young rats (Table 6.1). In contrast to IFM, no age-dependent changes in ascorbate or GSH content were observed in SSM (Table 6.1). The mitochondrial GSSG levels were generally higher in IFM in comparison to SSM regardless of age (Table 6.1). Even in young animals, a greater proportion of total GSH (GSH+2GSSG) in IFM were present as GSSG when compared to SSM from same animals ( $p < 0.05$ ; Table 6.1). In aged rats, the difference in the GSH redox status between IFM and SSM became more pronounced; IFM exhibited a 500% increase

( $p < 0.05$ ) in GSSG levels (Table 6.1) when compared to SSM from the same animals. These results suggest that the capacity to maintain thiol redox homeostasis is lower in IFM than in SSM.

The age-dependent changes in GSH redox status were only present in the IFM. Direct comparison between IFM isolated from young and old hearts show that the percentage of oxidized GSH almost doubled with age ( $p < 0.02$ ; Table 6.1). In contrast, no age-related differences in GSH and GSSG levels were observed in SSM. The selective alteration in the GSH to GSSG redox couple suggests that IFM are either exposed to a greater level of oxidative stress and/or exhibit a more limited reductive capacity with age. It is important to note that no significant differences in total mitochondrial GSH concentrations were observed in either IFM or SSM isolated from young and old rats. Thus, these results suggest that IFM exhibit impaired ability to maintain a reduced GSH pool rather than a loss in GSH uptake.

#### *Age-associated changes in mitochondrial antioxidant enzyme activities*

The age-related changes in mitochondrial antioxidant and thiol redox status suggest that only IFM from old rats may be in an increased pro-oxidant state. The mitochondrial redox status is largely determined by reducing equivalents, such as NAD(P)H, GSH, and ascorbate. More importantly, ascorbate and GSH are primarily maintained in a reduced state by thioredoxin (TrxR), glutaredoxin (GrxR) and GSSG reductases (GR). Therefore, we sought to determine whether alterations in any of these enzymes could account for the changes in antioxidant redox status observed.

Consistent with previous results, IFM and SSM from young rats showed no apparent differences in TrxR, GrxR or GR activities (Table 6.2).

The selective age-related loss in ascorbate and GSH levels in IFM directly implicate potential alterations in GrxR and GR. In agreement with this notion, IFM from old rats displayed a significant 33% and 44% decline in GrxR and GR activities in comparison to IFM from young rats (Table 6.2). Despite a marked loss in GrxR and GR activities, no apparent change in TrxR activity were observed between IFM from young and old animals (Table 6.2). These results indicate that the activities of enzymes directly involved in the reduction of ascorbate and GSH are compromised in IFM with age. Moreover, the conservation of TrxR activity alone may not be sufficient to compensate for the age-related loss of GrxR and GR activities in IFM.

The GrxR and GR activities in SSM appears to be unaffected by age (Table 6.2). Interestingly, we observed that SSM in old rats had a two-fold increase in TrxR activity relative to SSM from young rats, suggesting a potential compensatory effect (Table 6.2). These results demonstrate that the age-related loss in the capacity to maintain a normal antioxidant and thiol redox balance declines only in IFM.

#### *Age-associated changes in Complex IV activity*

The age-related enhancement of pro-oxidant conditions seen in IFM may result in oxidative damage to mitochondrial proteins, leading to impaired mitochondrial function. To test this hypothesis, Complex IV activity in IFM and SSM from both young and old rats were determined. Results show that there were no significant

differences in Complex IV activities between the two mitochondrial subpopulations isolated from young rats (Figure 6.2). Comparing Complex IV activity in mitochondria from young and old rats showed that Complex IV activity declined by approximately 40% ( $p < 0.005$ ) in IFM, while no age-associated differences were evident in SSM (Figure 6.2). These results indicate that the increased pro-oxidant environment in IFM of the aging rat heart is associated with the decline in Complex IV activity.

#### *Age-dependent changes in 4-HNE modified mitochondrial electron transport chain*

The oxidant-rich milieu combined with high levels of unsaturated lipids in mitochondria creates ideal conditions for generation of lipid-derived reactive aldehydes, such as 4-hydroxynonenal (4-HNE) and their potential to conjugate proteins, including Complex IV. This is also exemplified by *in vitro* experiments where incubating 4-HNE results in adduction and inactivation of Complex IV [168]. Thus, we determined whether the decrease in Complex IV activity was associated with increased oxidative damage by quantifying the extent of 4-hydroxynonenal adducted to Complex IV. Mitochondrial complexes were separated by BN-PAGE and probed for 4-HNE adducts using anti-4HNE polyclonal rabbit antisera. Results showed no differences in levels of 4-HNE adducted to Complex IV in the two subpopulations of mitochondria in young animals (Figure 6.3). However, IFM isolated from old rats exhibited a four-fold increase ( $p < 0.005$ ) in 4-HNE modified Complex IV when compared to IFM from young rats (Figure 6.3). No extensive age-related increases in

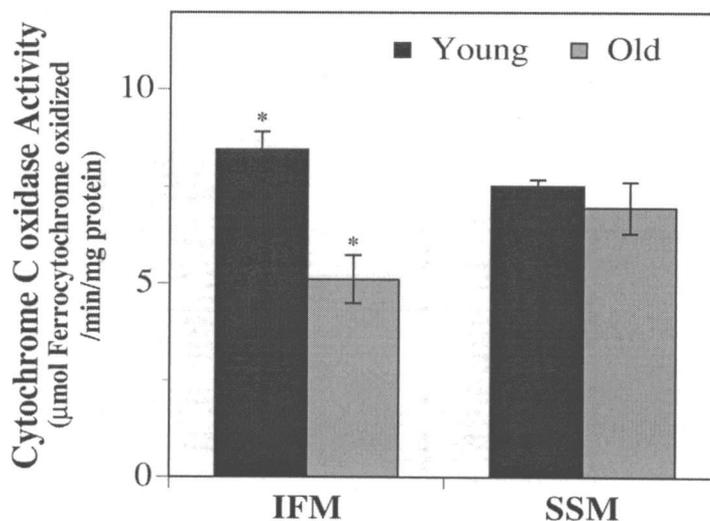
4-HNE modified Complex IV were observed in SSM. Thus, aging results in a selective increase in oxidative modification of Complex IV.

**Table 6.2 Age-associated changes in mitochondrial Thioredoxin and GSH related enzyme activities.**

	Young		Old	
	IFM	SSM	IFM	SSM
<b>Thioredoxin Reductase</b>	13.61±2.14	11.98±1.61	11.33±1.16	24.78±2.80
<b>GSSG Reductase</b>	19.28±0.86*	19.32±2.16	12.99±0.91*†	20.30±2.00†
<b>Glutaredoxin Reductase</b>	7.02±0.77*	6.46±0.49	3.96±0.44*†	6.78±1.11†

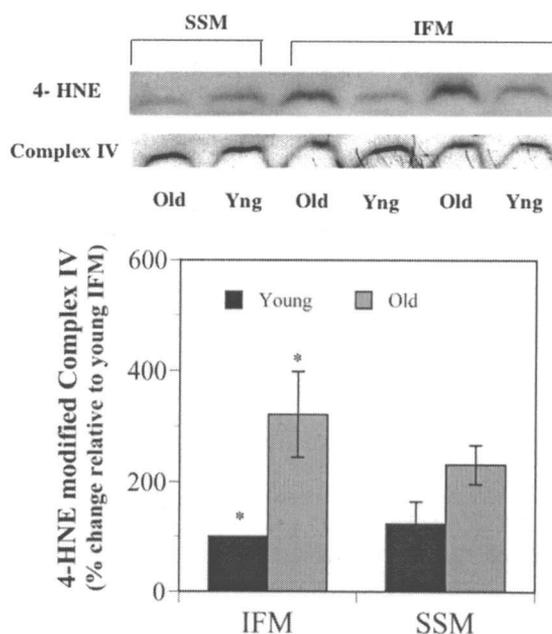
Units are nmol/min/mg protein and values are expressed as Mean±SEM

Activities of mitochondrial thioredoxin, glutaredoxin and GSSG reductase in IFM and SSM from young and old rats were measured as described in the methods. The results show a significant age-related loss in both GSH and glutaredoxin reductase activity in IFM isolated from old versus young animals. In contrast, thioredoxin reductase activity in IFM did not show any significant loss with age. Moreover, thioredoxin reductase activity increased by two-fold in SSM isolated from old animals. The results are expressed as mean ± SEM. (\* denotes significant (p<0.05) difference between identical mitochondrial preparations from young and old rats while † denotes differences between IFM and SSM within the same age-group)



**Figure 6.2** Age associated decline in Complex IV activity is only observed in IFM isolated from old rats.

To assess the functional consequence to the increased oxidative damage seen in IFM isolated from old rats, the maximal Complex IV activity was determined as described in the materials and methods. Results show a significant decline in detergent-solubilized Complex IV activity only in the IFM isolated from old (N=8) vs young (N=5) rats. Results are expressed as mean  $\pm$  SEM. (\* denotes significant ( $p < 0.05$ ) difference between identical mitochondrial preparations from young and old rats while † denotes differences between IFM and SSM within the same age-group)



**Figure 6.3. Age-related increase in the 4-HNE modified mitochondrial electron transport chain.**

Blue-native gel electrophoresis (BN-PAGE) was performed to separate the mitochondrial electron transport chain complexes from young and old animals. Proteins were transferred to PVDF membranes and were probed with polyclonal rabbit anti-sera as described. Results show a marked age-related increase in the steady state levels of 4-HNE modified Complex IV in IFM isolated from old (N=4) compared to young (N=4). Results are expressed as % increase relative to young IFM. Statistical significance was determined by ANOVA with Tukey's post-hoc test. (\* denotes significant ( $p < 0.05$ ) difference between identical mitochondrial preparations from young and old rats while <sup>†</sup> denotes differences between IFM and SSM within the same age-group)

## 6.5 Discussion

Mitochondrial decay and an accompanying increase in mitochondrial oxidant generation may be an important underlying cause of cardiac decline with age [157]. Previously, we described how aging cardiac myocytes exhibit increased mitochondrial oxidant generation and lowered antioxidant capacity, which leads to a heightened accumulation of oxidative damage to DNA [158]. We have now extended these observations to show that the age-associated increase in mitochondrial oxidant production and oxidative damage is not uniform but occurs almost exclusively in IFM. The implications of these results are profound. Close physical associations of IFM with myofilaments suggest that loss of IFM may adversely affect muscle contractility by limiting ATP required for myosin ATPases. Moreover, increased oxidant generation of IFM could promote mitochondrial-derived oxidant injury to myofilaments, leading to increased fibrosis and/or fiber rearrangement. Lastly, IFM dysfunction may initiate apoptosis and myocardial loss, ultimately decreasing the contractile capacity of the heart.

It is surprising that IFM are so markedly affected with age relative to SSM. IFM and SSM cannot be distinguished either structurally or morphologically; however, they occupy unique locales within the cell [18]. It is notable that no differences in oxidative stress parameters were observed between these subpopulations obtained from young rats, suggesting that isolation procedures did not affect the analysis of oxidative stress. The protease treatment during IFM isolation may increase

the possibility for selective damage to IFM, which, in turn, could confound results. However, this potential problem was addressed by Palmer and co-workers who found that the exposure of SSM to identical isolation procedures used for IFM does not affect their function or morphology [161]. Thus, the differences in the age-related changes evident in IFM and SSM functions are likely accurate reflections of the aging process.

The exact mechanism(s) for the selective age-dependent decline in IFM are not known. Mitochondrial turnover may be different between IFM and SSM. A slower rate of degradation and/or production of IFM would enhance the probability of damage during the aging process. Aside from mitochondrial turnover, IFM may be more prone to damage due to its high respiratory activity in comparison to SSM. In young animals, the oxidative rates of IFM were found to be 1.4 to 1.7 times higher than in SSM [18,161]. The selective decline in mitochondrial antioxidant capacity as seen by the loss of ascorbate and GSH could further increase susceptibility for oxidative damage in IFM.

Previous studies using mixed populations of mitochondria have yielded differential results with respect to age-related increases in oxidative stress. Some studies report an increased oxidant generation with age [130], while others, including Drew and coworkers, using a SSM enriched preparation, observed no change in mitochondrial oxidant production [17,169,170]. Apparent discrepancies in the extent of age-related changes in mitochondrial oxidant appearance and oxidative stress may reflect the differences in mitochondrial isolation procedures and resultant

heterogeneity in the amount of IFM and SSM obtained in sample preparations. It is notable that when the age-related changes in the rate of mitochondrial oxidant generation were examined in intact cardiac myocytes, a significant increase in the rates of oxidant appearance was observed in cells isolated from old compared to young rats [158]. The preferential decline in IFM may be a major contributing factor for the heightened rate of oxidant generation with age.

The selective increase in the rate of oxidant generation in IFM may be due to a number of different factors. First, the previously reported lesions in Complex III are only present in the IFM [160,171-173]. Since Complex III is one of the primary sites for electron leakage, its decline would likely result in heightened production of reactive oxygen species [160]. In addition, decreased Complex IV activity may cause blockage of electron flow and indirectly influence the oxidant generation from Complex III [159]. Second, the depletion of ascorbate and GSH, which was only observed in IFM, could result in further loss of other antioxidants such as ubiquinol and  $\alpha$ -tocopherol [174] and increase oxidant appearance in IFM.

The decreased antioxidant levels and GSH redox status in IFM suggest an age-related alteration in mitochondrial redox environment. The maintenance of a normal mitochondrial redox state is largely dependent on thioredoxin, glutaredoxin and GSSG reductases [163,166,175,176]. These enzymes have wide substrate specificity and are critical for maintaining antioxidants and protein thiols in a reduced state. Since *de novo* synthesis of ascorbate and GSH does not occur in mitochondria [177], any lesions in one or all of these enzymes may result in compromised antioxidant capacity

and altered thiol/disulfide ratio. Our results indicate that the significant loss in ascorbate and GSH seen in IFM is likely due to lower glutaredoxin and GSSG reductase activities; thioredoxin reductase appears to be maintained. Although not examined directly, one potential reason for the selective loss of glutaredoxin and GSSG reductase activities may be due to differences in their susceptibility to oxidative inactivation. In support of this, a previous study by Starke and co-workers showed that both of these enzymes are more susceptible to oxidative inactivation when compared to thioredoxin reductases [178].

Results show only IFM display an age-related loss in Complex IV activity, which was associated with an increase in 4-hydroxynonenal (4-HNE) adductions to Complex IV. Due to the close physical association with cardiolipin [172,179,180], Complex IV is especially susceptible for modification by 4-HNE. Cardiolipin is an excellent substrate for the formation of 4-HNE because it is largely composed of linoleic acid (C18:2), which is easily oxidized. Previous studies with rodents show a significant age-related loss in mitochondrial cardiolipin levels, which may occur due to oxidative damage and/or removal [181,182]. The selective increase in 4-HNE modified Complex IV in IFM may also be due to the significant age-related decline in GSH levels. The conjugation of free reactive 4-HNE with GSH represent one of the major route of elimination, hence decreased GSH content may lead to the accumulation of 4-HNE [183]. In contrast to current work, a study by Moghaddas and co-workers reported no apparent age-related changes in cardiolipin content, nor found

any increase in 4-HNE modified electron transport chain complexes [180]. The reasons for these discrepancies are not clear.

It is not clear whether the increased 4-HNE modification of Complex IV is a direct cause for the decrease in its activity. *In vitro*, exposures to 4-HNE deactivates a number of mitochondrial proteins, such as glucose-6-phosphate, pyruvate and  $\alpha$ -ketoglutarate dehydrogenases and the adenine nucleotide exchanger [156,184-186]. Similarly, multiple subunits on Complex IV (subunits II, IV, Vb, VIIa, VIIc and VIII) are readily modified by 4-HNE and lead to its inactivation [168]. Further studies are needed to determine the functional implications of our current findings.

IFM dysfunction may be closely linked to the age-related alterations in myocardial relaxation rate and calcium regulation [151,187]. Disruptions in IFM may limit ATP, which is critical for the dissociation of actin from myosin, affecting both systolic contraction and diastolic relaxation [147]. Alternatively, IFM-associated loss in ATP synthesis may compromise cytosolic calcium clearance. This, in turn, could depress force-generating capacity and increase cardiac stiffness observed with age [188].

Our results show that aging results in the selective depletion of antioxidant capacity, increased oxidative modification and a marked decline in Complex IV activity in IFM. Further studies are needed to assess whether age-dependent loss of myocytes is related specifically to increased damage seen in IFM.

## **6.6 Acknowledgement**

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## **Chapter 7.**

**Age-related decline in Nrf2-dependent glutathione synthesis:**

**Improvement by (*R*)- $\alpha$ -lipoic acid treatment**

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and Tory M. Hagen

## 7.1 Abstract

One of the hallmarks of aging is a decline in steady-state antioxidant levels. Glutathione (GSH) declines in the aging rat liver by  $35\pm 5\%$ ; however, feeding 0.2% (w/w) (R)- $\alpha$ -lipoic acid (LA) for 2 weeks reverses this loss. The mechanism(s) of GSH decline and its restoration by LA treatment may be due to decline in levels or activity of  $\gamma$ -glutamylcysteine ligase ( $\gamma$ -GCL), the rate-controlling enzyme in GSH synthesis. To examine whether there was also a decline in protein content, levels of catalytic ( $\gamma$ -GCLC) and modulatory ( $\gamma$ -GCLM) subunits were determined.  $\gamma$ -GCLC and  $\gamma$ -GCLM declined by 47 and 52 % respectively ( $p=0.001$ ). Treatment with LA (40 mg/kg bw) over 48 h reversed the age-related loss in  $\gamma$ -GCL activity. The basal expression of  $\gamma$ -GCL is primarily regulated at the transcriptional levels by Nrf2, a transcription factor belonging to Cap and Collar leucine b-ZIP family of proteins. We have observed that the nuclear levels of Nrf2 decline dramatically with age (40% loss;  $p<0.05$ ). Results from electrophoretic mobility shift assay reveal a significant reduction in the ARE binding activity with age. Interestingly, treatment with LA potently enhanced Nrf2 nuclear translocation and improved ARE binding activity. These results suggest the age-related decline in GSH levels is, in large part, due to Nrf2-dependent transcriptional deregulation of  $\gamma$ -GCL and LA reverses this loss.

## 7.2 Introduction

One of the hallmarks of aging is the loss of cellular homeostatic mechanisms, which renders the body more vulnerable to a variety of oxidative, toxicological and pathological insults [1,189,190]. Nowhere is this loss more exemplified than in the age-related decline in hepatic glutathione (GSH) levels. GSH is the principal low molecular weight thiol antioxidant, which also participates as a co-substrate for a variety of antioxidant and anti-xenobiotic (Phase II) enzymes [73,191-193]. Decline in constitutive GSH levels adversely affects cellular thiol redox balance and potentially leaves the cell susceptible to oxidative stress and xenobiotic insult. Conversely, increasing GSH steady-state levels and/or its rate of synthesis confers enhanced protection against oxidative stress. Due to the central role of GSH in cellular antioxidant defenses, the induction of enzymes required for its synthesis represent a key adaptive response to oxidative stress. Under normal conditions, exposure to pro-oxidants decreases cellular GSH and redox state, which are averted by increasing cellular GSH synthetic capacity [194,195]. One of the intriguing aspects of aging is that despite elevated basal levels of oxidative stress, the predicted increase in GSH or its synthetic enzymes are largely absent in various aging models. The lack of cellular compensatory response to loss in GSH and pro-oxidant stature in aging cells suggest that the coordination of cellular antioxidant defenses may be altered with age.

The synthesis of GSH from its constituent amino acids involves the actions of two ATP requiring enzymes, the  $\gamma$ -glutamylcysteine ligase ( $\gamma$ -GCL) and GSH

synthetase (GS). The sustained increase in GSH is primarily achieved by increasing the activity or protein levels of  $\gamma$ -GCL, the rate-regulating enzyme in the GSH synthetic pathway.  $\gamma$ -GCL exists as a heterodimer composed of a catalytic ( $\gamma$ -GCLC; 73 kDA) and a modulatory ( $\gamma$ -GCLM; 30 kDA) subunit. While  $\gamma$ -GCLC retains all of the catalytic activity,  $\gamma$ -GCLM improves its catalytic activity by lowering  $K_m$  for glutamate and altering the negative feedback inhibition by GSH [196].

The enhancer regions of both  $\gamma$ -GCLM and  $\gamma$ -GCLC contain multiple putative sites for binding transcription factors, such as nuclear factor kappa B (NF- $\kappa$ B), SP-1, activator protein-1 and 2 (AP-1 and 2), metal response (MRE), and antioxidant response elements (ARE) [197-199]. Among these, the basal and inducible expression of the two components of  $\gamma$ -GCL appears to be mediated via AREs [200-202]. The ARE is a cis-acting enhancer sequence that mediates transcriptional activation of Phase II detoxification enzymes that are critical for maintaining cellular redox status and protecting against oxidative damage [203]. Therefore, the potential age-related loss in transcriptional regulation of  $\gamma$ -GCL may represent a global decline in Phase II defense system with age.

Recent studies show that NF-E2 related factor-2 (Nrf2) is the principal transcription factor that regulates ARE-mediated gene transcription [204,205]. Direct evidence for this is provided by the observation that Nrf2-null mice exhibit reduced basal expression of  $\gamma$ -GCL and other phase II detoxification enzymes [204,206-208]. The striking similarities in decreased stress tolerance observed between aging and

Nrf2 null mice raise the question as to whether disruptions in Nrf2 signaling pathway occur during aging.

The potential involvement of Nrf2 disruption in aging suggests that dietary agents that induce Nrf2-dependent transcriptional activation may improve the age-related decline in stress tolerance. Indeed, thiol reactive substances such as 3H-1,2-dithiole-3-thione (D3T), pyrrolidine dithiocarbamate (PDTC)[209], and sulforaphane act as a potent chemopreventive agents that increase cellular GSH and Phase II response. Likewise, we have previously observed that supplementation with (*R*)- $\alpha$ -lipoic acid, a natural dithiol compound, to old rats markedly improves resistance to alkyl peroxides and tissue GSH status [193]. Thus, the aims this study was to determine 1) whether the decline in GSH status that we observed during aging is due to the loss of Nrf2-dependent regulation of GCL expression, and 2) whether LA reverses the loss in hepatic GSH in aging rats by acting as an inducer of Nrf2-dependent gene transcription.

### 7.3 Materials and Methods

#### *Materials*

(*R*)- $\alpha$ -lipoic acid (LA) was a gift of Asta Medica (Frankfurt, Mainz, Germany). Rabbit polyclonal antibodies to  $\gamma$ -GCL heavy and light subunits were obtained as reference [73]. All other antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All high performance liquid chromatography (HPLC) solvents were HPLC grade reagents from Fisher Scientific (Houston, TX). All other chemicals were reagent grade or the highest quality available from Sigma.

#### *Animals*

Rats (Fischer 344, virgin male, outbred albino), both young (2 to 5 months: N=20) and old (24 to 28 months, N=20; National Institute of Aging animal colonies), were acclimatized in the Oregon State University animal facilities for at least one week prior to experimentation. Animals were maintained on standard chow diet. Both food and water were given ad libitum.

LA (40 mg/ml) was dissolved in 2 M NaOH solution containing 154 mM NaCl and pH was adjusted to 7.4 with concentrated HCl. LA solutions were sterile-filtered and made fresh each day of use. LA (40 mg/kg body wt.) was administered by *i.p.* injection. To reduce diurnal variations, animals were sacrificed between 10 to 11 am each morning.

Rats were anesthetized with diethyl ether and a midline incision was made in the abdomen. Heparin (0.4 mg/ml) was injected via the femoral vein and Hank's balanced salt buffer (pH 7.4; HBSS) was perfused through the hepatic portal vein for 5 min to remove blood. Livers were quickly removed and washed twice in ice cold HBSS.

#### *Glutathione (GSH) analysis*

Hepatic GSH and GSSG concentrations were determined according to the methods of Farris and Reed [210]. Liver tissue (200 mg) was minced and homogenized in 10% (w/v) perchloric acid (PCA) containing 5 mM EDTA. After centrifugation, 170  $\mu$ l of the supernatant containing internal standard ( $\gamma$ -glutamyl-glutamate; 100  $\mu$ M final) was mixed with 50  $\mu$ l of iodoacetic acid (100 mM dissolved in 0.2 mM m-cresol purple) and pH adjusted to 10 by using KOH-KHCO<sub>3</sub> buffer (2M KOH:2.4 M KHCO<sub>3</sub>). Samples were placed in the dark at room temperature for 1 h. Following completion of S-carboxymethyl derivatization of free thiols, equal volume of 1% 1-fluoro-2,4-dinitrobenzene was added and incubated overnight in the dark at room temperature. Fifty  $\mu$ l of samples were separated by HPLC [210] and detected using a Bioanalytical Systems (BAS; West Lafayette, IN) UV-116A spectrophotometer with the absorbance set at 365 nm. Quantitation was obtained by integration relative to internal standard.

### *Measurement of $\gamma$ -glutamylcysteine ligase ( $\gamma$ -GCL) activity*

Hepatic  $\gamma$ -GCL activity was detected as described previously [73]. Briefly, tissues were homogenized in 0.25 M sucrose containing 1 mM EDTA, 20 mM Tris-HCl (pH 7.4), and 1 % (v/v) protease inhibitor cocktail P8340 (Sigma Life sciences). The homogenates were centrifuged at  $3000 \times g$  for 10 min at 4 °C. The supernatants were centrifuged at  $20,000 \times g$  for 30 min. These samples were filtered in microcon-10 (Amicon) tubes for 45 min at 4 °C and washed twice with 0.2 ml of lysis solution (0.1 M Tris-HCl, pH 8.2, 150 mM KCl, 20 mM MgCl<sub>2</sub>, 2 mM EDTA). Proteins were quantified by using a standard Lowry assay. The enzyme reaction was initiated by adding protein (0.5 mg/ml) to reaction buffer containing 20 mM L-glutamic acid, 5 mM cysteine, 5 mM dithiothreitol, 10 mM ATP, 0.1 M Tris-HCl (pH 8.2), 150 mM KCl, 20 mM MgCl<sub>2</sub>, 2 mM EDTA and 0.04 mg/ml acivicin. The samples were incubated in waterbath at 37 °C for 45 minutes. The reactions were stopped by mixing 150  $\mu$ l of sample with an equal volume of 10% PCA. The amount of  $\gamma$ -GCL formed was detected using GSH analysis method as described above. Quantitation was obtained by integration relative to  $\gamma$ -GCL external standard.

### *Western blotting analysis*

Tissues were homogenized and processed as described for the analysis of  $\gamma$ -GCL activity. An aliquot of tissue homogenate (100  $\mu$ l) was used for determining  $\gamma$ -GCL protein content by Western blotting as described before [73].  $\gamma$ -GCLC and  $\gamma$ -

GCLM were identified according to molecular weight markers. Relative densities of the bands were digitally quantified using NIH image analysis software.

#### *Nuclear NF-E2 related factor 2 (Nrf2)*

Nuclear extracts were prepared from liver tissue by the method of Dignam and co-workers [211]. 40 µg protein was loaded in each well of a 12% Tris-HCl polyacrylamide gel (BioRad Laboratories, CA). The proteins were transferred to a nitrocellulose membrane (Amersham, Inc.) and probed with anti-Nrf2 at a 1:2000 titer. Chemiluminescent detection was done by ECL kit from Amersham.

#### *Electrophoretic mobility shift assay (EMSA)*

Binding to ARE was determined using an electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described earlier. All gel-shift assays were performed for 3 sample replicates in each group. Synthetic double-strand oligonucleotide probe for ARE (5'-CTACGATTTCTGCTTAGTCATTGTCTTCC-3') (Panomics, Inc., CA) was end-labeled using [ $\gamma$ -<sup>32</sup>P] (Amersham) and T4 polynucleotide kinase (Promega). Binding reactions containing equal amounts of protein (5µg) and labeled oligonucleotide probes were performed for 20 min at room temperature in binding buffer (4% glycerol, 1mM MgCl<sub>2</sub>, 0.5 mM EDTA, pH 8.0, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris). Specific binding was confirmed using 100-fold excess unlabeled ARE oligonucleotide as a specific competitor and by supershift assays in which 1 µl anti-Nrf2 (Santa Cruz Biotechnology, CA) was incubated with

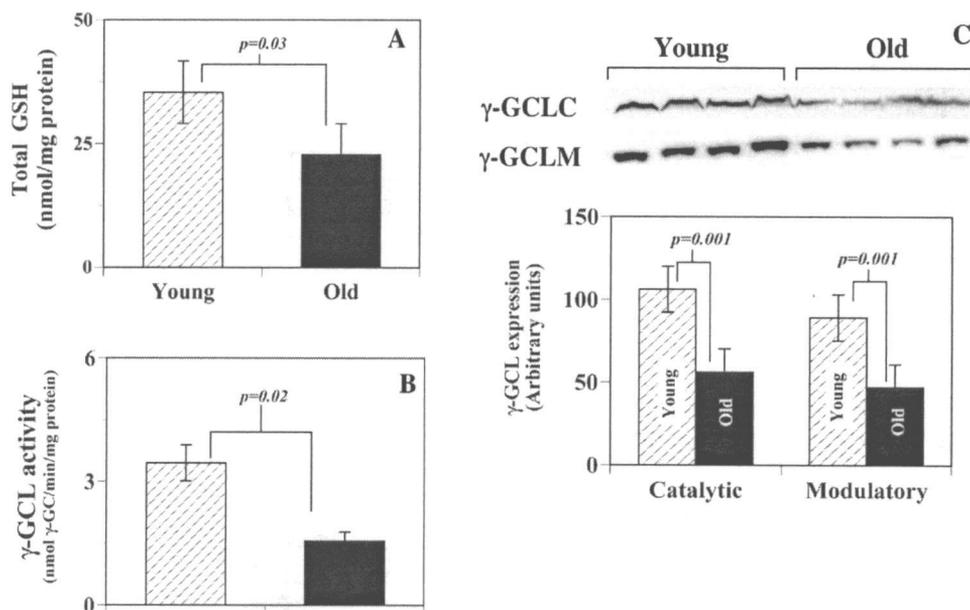
the protein at room temperature for 20 minutes immediately prior to the reaction. Protein-DNA complexes were separated using 6% non-denaturing polyacrylamide gel electrophoresis followed by radiography to detect the degree of retardation produced by binding to the probe.

## 7.4 Results

### *Age-associated changes in GSH levels and synthetic capacity*

To establish the extent of age-related changes in GSH levels, the hepatic GSH levels were determined in young and old rats. Compared to young rats, old animals exhibited a significant 35% decline ( $p=0.03$ ) in total GSH levels in the liver (Figure 7.1A). The loss in total GSH may be partly due to the formation of mixed disulfides with proteins. To ascertain the amount of GSH lost due to direct conjugation with proteins, liver homogenates depleted with endogenous GSH were incubated with 10 mM dithiothreitol (DTT) to release GSH bound to proteins. In young animals, the amount of protein-bound GSH was  $11.63 \pm 1.52$  pmol/mg tissues. With age, the concentration of GSH bound to proteins increased to  $19.82 \pm 5.15$  pmol/mg tissue, a 40% increase ( $p=0.02$ ). Despite this increase, the concentrations of GSH lost due to conjugation were proportionally minor in comparison to the age-associated differences in total hepatic GSH levels. Thus, the age-associated increase in S-glutathiolation is not a significant contributing factor to the loss of GSH seen during aging.

To discern whether age-related loss in GSH concentrations in the liver was due in part to the diminished GSH synthetic capacity, the protein levels and activities of  $\gamma$ -GCL were examined. Results show a 53% loss in tissue  $\gamma$ -GCL activities in old animals when compared to young ( $p=0.02$ ; Figure 7.1B). Since  $\gamma$ -GCL is a heterodimer, the loss in total  $\gamma$ -GCL activity may be due to the low expression of



**Figure 7.1.** Age-related decline in total hepatic GSH is due to loss in  $\gamma$ -GCL activity and expression.

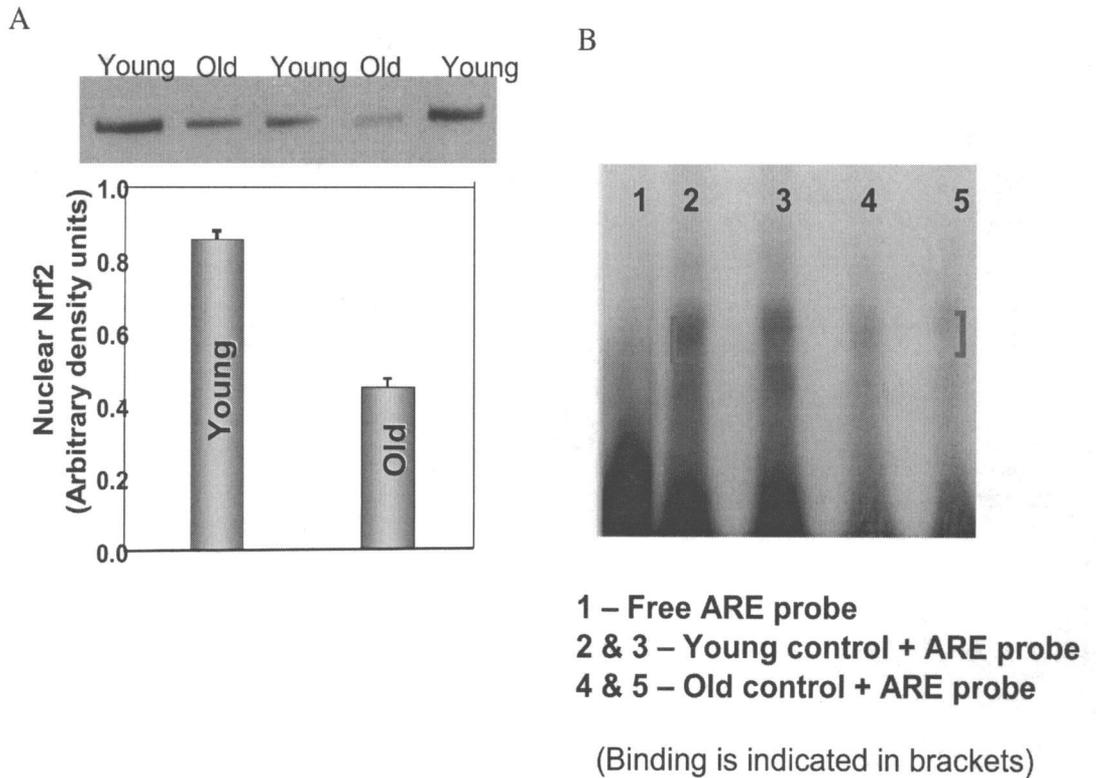
Hepatic GSH levels in young (3 mo; N=4) and old (24 mo; N=4) F344 rats are shown in Panel A. Results show a 35% decline in total GSH (GSH+2[GSSG]) in old relative to young rats. Panel B shows the age-dependent changes in the hepatic  $\gamma$ -GCL activity. A profound age-related decline in  $\gamma$ -GCL activity was observed in old versus young animals. The diminished  $\gamma$ -GCL activity in old rats was accompanied by approximately 42% and 52% decline in the levels of GCLC and GCLM, respectively. Results are expressed as mean  $\pm$  SEM.

catalytic or modulatory subunits. To assess how aging affects the steady-state levels of these subunits, western analysis using tissue extracts used for  $\gamma$ -GCL activity assay were performed. Results show an approximately 47% ( $p=0.001$ ; Figure 7.1C) decrease in  $\gamma$ -GCLC expression in old relative to young animals.  $\gamma$ -GCLM levels in old animals were also found to be lower than that of young animals by 52% ( $p=0.001$ ; Figure 7.1C). These results support the notion that the age-related attenuation of hepatic GSH content is largely due to loss in the activity of  $\gamma$ -GCL. The loss of  $\gamma$ -GCL activity is likely a reflection of the diminished expression of both  $\gamma$ -GCLC and  $\gamma$ -GCLM in the aging rat liver.

#### *Age-related decline in Nrf2 and Antioxidant Response Element (ARE) binding*

The decrease in  $\gamma$ -GCLC and  $\gamma$ -GCLM protein levels suggests that their transcription may be altered with age. Due to the central role of Nrf2 in regulating ARE-mediated gene transcription, we next examined whether the basal nuclear Nrf2 levels are adversely affected during aging. To determine the extent of age-related changes in basal nuclear Nrf2 levels, western blot analysis was performed using hepatic nuclear extracts from young and old rats (Figure 7.2). In comparison to young, old animals exhibited approximately 40% lower basal nuclear Nrf2 levels ( $p<0.05$ ) (Figure 7.2A). This decrease may significantly lower basal ARE-dependent transcription.

To assess the consequence of decreased Nrf2 levels to ARE binding activity, electrophoretic mobility shift assay was performed (Figure 7.2B). As predicted by



**Figure 7.2. Aged rats display a significant loss in nuclear Nrf2 content and ARE binding activity.**

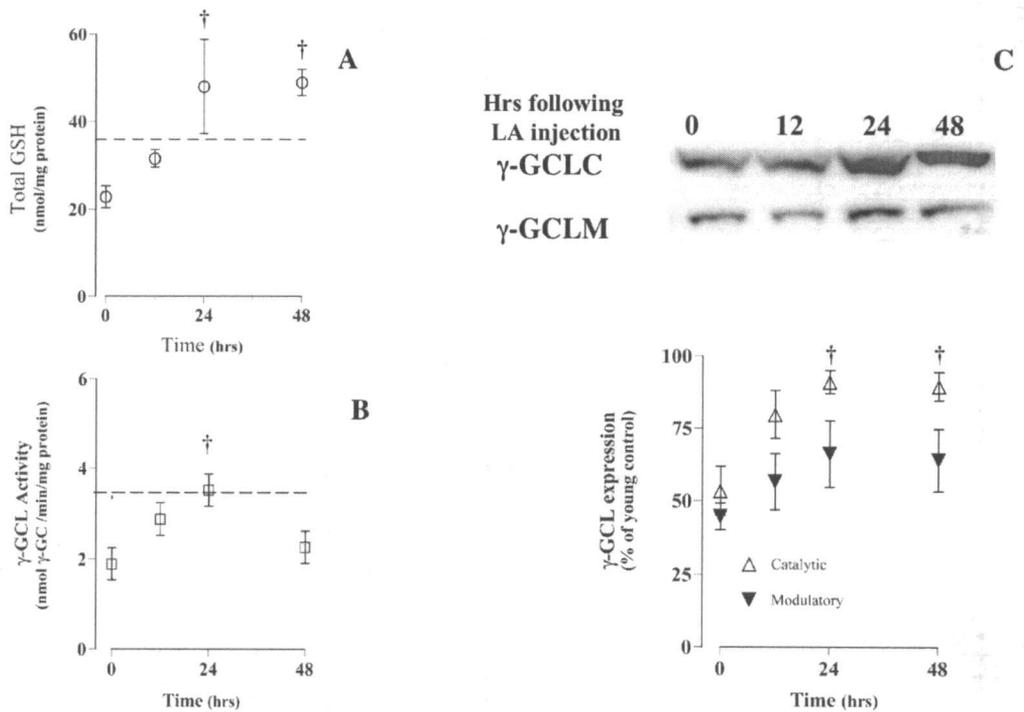
The hepatic nuclear Nrf2 levels in young and old rats are shown. Relative to young animals, old animals contain significantly lower constitutive levels of Nrf2 (Panel A). The decreased nuclear Nrf2 levels were associated with a marked loss in ARE binding activity in old rats when compared to young (Panel B).

decreased Nrf2 protein content, the basal ARE binding activity was reduced in nuclear extracts obtained from old versus young rats. These results suggest that the transcription of ARE-encoding proteins is decreased with age.

*Treatment with LA reverses the age-related loss in GSH*

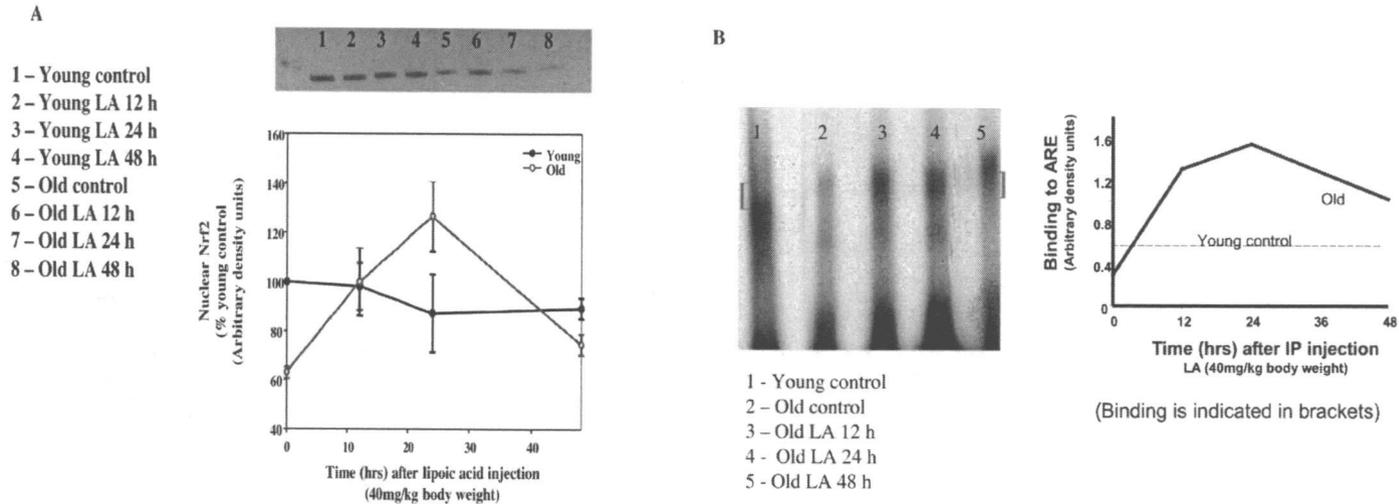
Previous studies indicate that LA supplementation for two weeks reverses the age-associated loss in basal GSH levels of isolated hepatocytes [193]. Similar dithiol compounds, such as isothiocyanates, sulforaphane, and pyrrolidine dithiocarbamate, have been demonstrated to induce Nrf2. These studies suggest that the mechanisms of LA-mediated improvement in GSH status are dependent on LA-induced Nrf2 activation and subsequent increase in  $\gamma$ -GCL expression. To examine this possibility, young and old rats were treated with either LA (40 mg/kg body weight) or an equal volume of saline at 12 and 24 hrs by *i.p.* injection, and groups of animals were sacrificed at times indicated. Results show that a short-term treatment with LA was sufficient to normalize the hepatic GSH concentrations within 24 hrs following treatment (Figure 7.3A). No significant differences in GSH levels were observed between saline- and LA-treated young animals (data not shown).

To assess whether improved rate of GSH synthesis is responsible for the LA-mediated reversal in hepatic GSH, the changes in hepatic  $\gamma$ -GCL activity were monitored. Results show that within 24 hrs following LA administration, the  $\gamma$ -GCL



**Figure 7.3.** LA treatment reverses the age-related decline in GSH synthetic capacity and reverses the loss of hepatic GSH levels in aging rats.

Changes in hepatic GSH,  $\gamma$ -GCL activity and the expression of GCLM and GCLC were examined in old rats treated with saline vehicle (control) or LA (40 mg/kg bw), at the time points indicated. Results show a marked improvement (190% increase at 24 hrs following LA injection;  $p=0.05$ ) in hepatic GSH content in LA treated old rats relative to the age-matched controls (Panel A). Similarly, old rats treated with LA displayed a significant 200% increase in  $\gamma$ -GCL activity within 24 hours following injection (Panel B). The dotted lines in Panel A and B denotes mean experimental values seen in young animals. The increased activity of  $\gamma$ -GCL in old LA treated rats was due to a marked increase in the levels of GCLC (Panel C). The differences seen in GCLM following LA treatment were not statistically significant (Panel C). Results are expressed as the mean  $\pm$  SEM and  $\dagger$  denotes groups that are significantly ( $p<0.05$ ) different from control animals.



**Figure 6.4. LA attenuates the loss in nuclear Nrf2 levels and increases its ARE binding activity.**

To determine whether LA-mediated increase in GCL activity and expression is associated with the increase in nuclear Nrf2 levels, the hepatic nuclear Nrf2 levels were determined in young and old rats treated with LA for indicated times. Results show LA administration strongly increases nuclear Nrf2 levels in old animals (Panel A). In contrast, young Nrf2 levels were not affected by LA treatment (Panel A). In panel B, the electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts obtained from same animals. The results show LA treatment effectively increased ARE binding that LA-induced increased ARE binding activity in old rats after 24 hrs following treatment (Lanes 2 versus 5). Similarly, young rats also exhibited a small increase in ARE binding activity within 24 hrs following LA administration. These results indicate that LA-mediated reversal in hepatic GCLC is associated increased ARE activation.

activity increased by almost doubled in old rats ( $p < 0.05$ ; Figure 7.3B). The LA-mediated increase  $\gamma$ -GCLC was abolished after 48 hrs, suggesting the depressed activity at this time point was due to feedback inhibition by increased GSH levels (Figure 7.3B). In contrast, LA treatment did not alter  $\gamma$ -GCL activity in young rats (Data not shown).

The age-related loss in  $\gamma$ -GCLC and  $\gamma$ -GCLM directly correlated with the decrease in  $\gamma$ -GCL activity and tissue GSH content. The reversal in hepatic GSH upon LA administration may be dependent on increased protein synthesis. To determine this, the protein levels of  $\gamma$ -GCLC and  $\gamma$ -GCLM in samples used for  $\gamma$ -GCL activity assays were determined. Paralleling the changes in enzymatic activity, LA reversed the age-related decline in  $\gamma$ -GCLC levels within 24 hrs (1.7-fold increase;  $p < 0.05$ ; Figure 7.3C). In contrast to  $\gamma$ -GCLC, no significant increase in  $\gamma$ -GCLM was observed in old LA-treated animals (Figure 7.3C). These results suggest that although the loss in GSH levels with age was associated with diminished expression of both  $\gamma$ -GCLC and  $\gamma$ -GCLM, it appears that induction of  $\gamma$ -GCLC alone is sufficient for improving hepatic GSH status in the aging rat liver.

#### *Role of LA as an activator of Nrf2*

The strong association between loss in nuclear Nrf2 content and decreased expression of  $\gamma$ -GCL suggest that LA-induced activation of Nrf2 may be required for increased protein synthesis. To test this hypothesis, the changes in nuclear Nrf2 content were examined. Results show that LA treatment markedly increased nuclear

Nrf2 levels in the aging rat to levels similar to young animals (Figure 7.4A). Interestingly, the LA-induced increase in nuclear Nrf2 levels was not apparent in the young animals. This suggests that LA effect on Nrf2 translocation is age-specific. However, the lack of corresponding increase in  $\gamma$ -GCL expression and activity in young rats suggest the influence of another additional regulatory control.

Given the association between nuclear Nrf2 and ARE genes, the electrophoretic mobility shift assay was performed to evaluate Nrf2 binding to ARE sequence, using nuclear extracts from young and old rats, pre-treated with or without LA. The results show that LA treatment effectively increased ARE binding activity in old rats, at as early as 12 h, with a maximum at 24 hrs following treatment (Figure 7.4B, lanes 4-5 versus 2). These results are in full agreement with the maximum observed in the maximum  $\gamma$ -GCL activity (Figure 7.3B) and the maximal levels of Nrf2 (Figure 7.4A), indicating that LA-mediated reversal in hepatic  $\gamma$ -GCL is associated with enhanced ARE activation.

## 7.5 Discussion

The present study demonstrates for the first time that Nrf2-mediated transcriptional regulation becomes altered during aging. The disruption in basal Nrf2-mediated signaling provides the basis for the paradoxical decline in basal levels of tissue GSH and its synthetic machineries under enhanced pro-oxidant conditions of aging. Considering that over 200 antioxidant and phase II detoxification enzymes are regulated by the Keap1-Nrf2 pathway [212], the alterations in basal nuclear Nrf2 levels and ARE binding activity is likely to have broad impact on various aspects of cellular antioxidant and xenobiotic responses. Furthermore, the efficacy of LA in reversing the age-related decline in Nrf2-mediated expression of  $\gamma$ -GCL suggests that other known phase II inducers (such as sulforaphane) may also be effective in improving cellular defenses, which otherwise declines with age.

The loss of GSH content in aging tissues has been widely reported [73,193,213] and appears to be mitigated in large part by the decreased in levels and activities of  $\gamma$ -GCL [73,213]. The decreased  $\gamma$ -GCL protein and mRNA levels in several visceral tissues during aging suggest that this process is not a tissue specific phenomenon [73,213]. The upstream signaling events leading to  $\gamma$ -GCL are not completely understood yet. Interestingly, the sequence analysis of a 1.76 kb region of  $\gamma$ -GCLC and a 1.78 kb region of  $\gamma$ -GCLM from rats does not appear to contain the ARE that are present in humans. However, it should be noted that the functional element (ARE4) that mediates the effects of  $\beta$ -naphthoflavone is present in a region

upstream of those cloned from rats [197]. Thus, it is possible that identical functional elements could be present in the portion of rat  $\gamma$ -GCLC that has not yet been cloned. Furthermore, the rat  $\gamma$ -GCLM sequence also contains the consensus binding sites for TCF11, a transcription factor that belongs to the same class of CNC-bZip proteins as Nrf2. Due to the close homology between Nrf2 and TCF11, it is possible that the TCF11 consensus region may also act as the functional ARE site. Interestingly a recent study by Sekhar and co-workers showed that indomethacin treatment in rats caused a marked increase in  $\gamma$ -GCL expression by Nrf2 dependent mechanism [214]. This suggests a conserved function of Nrf2 in the activation of  $\gamma$ -GCL across various species including rats [215].

Recent studies using Nrf2 null mice, Nrf2 over-expression systems, and Nrf2 dominant-negative mutants show that this transcription factor is involved in both basal and inducible expressions of various ARE-containing genes including  $\gamma$ -GCLC and  $\gamma$ -GCLM [204,206-208,216-218]. With respect to diminished GSH synthetic capacity and stress response, the Nrf2 null mice share remarkable similarity to the senescent phenotype. Accordingly, it raises the possibility that similar deregulation of Nrf2-dependent transcription may occur during aging. Based on our experimental results (Figure 2), we confirmed that constitutive nuclear Nrf2 levels decline significantly in the aging rat liver. Moreover, our results also indicate that transcription factor binding to ARE declines with age.

The control of ARE-driven gene transcription by Nrf2 is a complicated process, with multiple layers of regulation, details of which are just now emerging.

Since Nrf2 does not form homodimers, it requires the presence of other transcription factors that belong to the cap and collar leucine bZIP family, such as c-Jun, small Maf, ATF4, and Fos [204,219-221]. A differential regulation of transcription of ARE encoding genes can be obtained by modulating other transcription factors. For example, the over-expression of c-Jun appears to enhance Nrf2-mediated activation of ARE genes while dimerization with Fra or JunB leads to its repression [205]. It is interesting to note that nuclear levels of JunB have been reported to increase with age [222]. Thus, the decline in Nrf2-dependent transcription may also involve alterations in the levels of other transcription factors that repress the ARE activation by Nrf2.

The age-related loss in basal nuclear Nrf2 levels shown in this work may be due to a number of factors. First, the total cellular transcription of Nrf2 protein may decline with age. A study by Kwak and co-workers showed that nuclear accumulation of Nrf2 requires new transcription [223]. Interestingly, it appears that the transcription of Nrf2 gene is subject to autoregulation. Thus, decreased Nrf2 expression may further repress its own transcription. In addition, the promoter regions of Nrf2 gene also contain Sp-1 binding domain, a transcription factor previously reported to decline with age [224]. The relative importance of these changes to Nrf2 gene transcription remains to be elucidated.

Additionally, the rate of Nrf2 turnover may be altered with age, either by decreasing its synthesis or accelerating its degradation. Two independent studies suggest that new protein synthesis is required for electrophile-mediated Nrf2 nuclear translocation [203,225]. These studies show that in absence of any inducers, Nrf2 has

a short half-life of approximately 20 min [225]. The proposed mechanisms suggest that the nuclear translocation of Nrf2 requires new protein synthesis and protection from rapid degradation. Nguyen and co-workers showed that the degradation of Nrf2 protein can be prevented if Nrf2 is phosphorylated via mitogen activated protein kinase/extracellular regulated kinase (MAPK/Erk) pathway [203]. In light of the reports that Erk activation decreases in aging hepatocytes [226-229], the decreased Erk activation may increase the basal rate of Nrf2 degradation, leading to lower constitutive nuclear levels. Currently, the contribution of Erk in mediating Nrf2 signaling in the context of aging has not been defined yet.

In the present study, a short-term LA treatment in old rats almost completely reversed the age-dependent loss in GSH synthetic capacity, resulting in the normalization of hepatic GSH status. The beneficial effects of LA on tissue GSH levels have been proposed to be dependent on the direct antioxidant role of DHLA [42]. However, considering the low concentration of free DHLA or LA present in tissues following LA treatment, it is unlikely that its antioxidant effect alone is sufficient to maintain the increased tissue GSH concentration [230,231]. Alternatively, the prolonged benefits associated with LA may be due to its ability to induce Nrf2-mediated transcription of  $\gamma$ -GCL. This notion is confirmed by our observation that LA potently induces nuclear Nrf2 translocation and subsequent ARE binding. This mechanism also applies to other dithiol compounds such as sulforaphane. Although sulforaphane does not directly exhibit antioxidant properties, it provides prolonged protection against oxidative stress by inducing Phase II enzymes

and GSH synthesis in cells by Nrf2 activation [232]. The LA effect on both Nrf2 activation and subsequent  $\gamma$ -GCL expression further supports our previous conclusion that the age-related alterations in Nrf2 may be responsible for the decline in GSH levels in the liver.

LA-dependent activation of Nrf2 may involve two potential mechanisms. Under basal conditions, Nrf2 remains largely bound to Keap1 (INrf2 in rats), a protein which belong to the kelch repeat superfamily of proteins. The Keap1 bound Nrf2 is rapidly degraded by ubiquitin-26S proteosomes. Studies show that liberation of Nrf2 from Keap1 occurs by two potential mechanisms. Keap1 contains 25 cysteine residues, out of which four are especially sensitive to oxidation [233]. The oxidation of these thiols on Keap1 causes subsequent conformational change that releases Nrf2. Alternatively Nrf2 can be released from Keap1 upon phosphorylation by kinases that are activated by oxidative or electrophilic stress [203]. Upon exposures to oxidants or thiol reactive compounds, Nrf2 is released from Keap1, which prevents its proteosomal degradation and allows it to translocate to the nucleus. As a dithiol compound, LA may directly liberate Nrf2 by interacting with critical thiols present in Keap1 [233].

In addition to direct thiol effects, LA may also initiate Nrf2 activation by induction of up-stream signal cascades. A cross-species comparison of Nrf2 amino acid sequence has identified several potential phosphorylation sites [234-236]. To date, three major signal transduction pathways have been implicated in regulation of the ARE, which include those mediated by the MAPK, PI3K, and PKC [203].

Previous studies show LA can induce PI3K pathway via direct activation of insulin receptor substrate –1 (IRS-1) [237]. The role of PI3K has been demonstrated in IMR-32 cells where the inhibition of PI3K by LY294002 abolished the induction of genes activated by t-butylhydroxyquinone, a potent inducer of Nrf2 [238]. Additionally, induction of PI3K also appears to regulate rearrangement of actin and enhance nuclear translocation of Nrf2 [239]. However, due to the extensive cross-talk that exists between different signaling cascades, LA may still induce Nrf2 nuclear accumulation by other non-PI3K dependent signaling pathways.

In summary, the evidence presented in this paper shows for the first time a definite association between aging and declined levels of GSH, diminished  $\gamma$ -GCL activity (in parallel with decreased expression of the catalytic and modulatory subunits), and lowered Nrf2 expression and binding to ARE sequence in rat liver. Remarkably, all these events can be effectively reversed by the administration of LA, modulating the parameters to return to the observed in young animals. The implications of this work open new avenues not only for further understanding of the aging process but also for possible strategies in its modulation by the administration of micronutrients.

## **7.6 Acknowledgement**

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## **Chapter 8**

### **General Conclusion**

## 8.1 General conclusion

The importance of dietary antioxidants for optimal health and for the prevention of chronic diseases is becoming widely recognized. Terms, such as “free radicals” and “oxidative stress” are now popularized in various commercials for products ranging from cosmetics to everyday food items. The increased public awareness of benefits associated with antioxidant intake is clearly reflected by the fact that Americans spent over 8 billion dollars on antioxidant supplements in 1995 (Commission on dietary supplement labels; 1997). Considering the enormous public interests in potential prophylactic use of antioxidants in maintaining health span, the elucidation of modes of antioxidant function under different scenarios of oxidative stress is an important step for proper implementation of antioxidant therapy.

Transition metals such as iron and copper are essential for maintaining normal metabolic functions. However, as discussed in chapters 2-5, excess accumulation of free redox active metals are potentially deleterious. The unique aspect of transition metal-ions chemistry is that they cause antioxidants, such as ascorbate to act as a pro-oxidant. Because transition metal toxicity is implicated in number of chronic diseases (e.g., Alzheimer’s, Parkinson’s, cancer and atherosclerosis), understanding the physiological relevance of the potential pro-oxidant interactions between antioxidants and transition metal-ions is central to understanding whether antioxidant supplementation can have undesirable effects.

In this dissertation, we directly addressed whether ascorbate acts as a pro- or anti-oxidant in the presence of redox-active transition metal ions and H<sub>2</sub>O<sub>2</sub>, using freshly isolated human plasma as a physiological fluid. The effects of different ascorbate concentrations on transition metal ion-dependent oxidation of plasma antioxidants, lipids and proteins in the presence or absence of H<sub>2</sub>O<sub>2</sub> were determined. Our results show that ascorbate (AA) does not act as a pro-oxidant towards lipids and proteins in human plasma *in vitro*, even under the most “oxidizing” conditions of the Udenfriend system (metal ion, ascorbate, H<sub>2</sub>O<sub>2</sub>). Thus, ascorbate in human plasma does not enhance protein and lipid oxidation, even in the presence of redox-active iron or copper and H<sub>2</sub>O<sub>2</sub>. To the contrary, our current and previous data [72] show that ascorbate strongly protects against metal ion-mediated lipid peroxidation in plasma *in vitro*, whereas  $\alpha$ -tocopherol is ineffective.

Based on the potent antioxidant role of AA in protecting against metal-driven oxidative stress, the age-related loss of tissue AA in the brain and the heart may limit the ability of these tissues to withstand pro-oxidant effects of excess transition metal accumulation (chapters 4-6). Indeed, restoration of tissue ascorbate levels in aging animals via treatment with LA dramatically lowered tissue levels of oxidative damage markers (chapters 4-6). Thus, the maintenance of normal ascorbate levels is likely to be a critical for the protection against age-related increases in oxidative stress.

LA has been referred to as a “metabolic antioxidant” based on its dual role as an antioxidant and as a required co-factor for  $\alpha$ -keto acid metabolism [42,103,157,240-242]). Indeed, previous studies using primary hepatocytes have demonstrated the potential use of LA in improving heightened levels of oxidative stress in aging rats [193,243]. In this dissertation, we extended these findings to show profound improvements in various indices of oxidative stress in post-mitotic tissues such as the heart and brain by LA treatment. Moreover, results presented in this dissertation provide new insights into the potential mechanisms underlying pharmacological effects of LA.

LA has been proposed to act as an efficient metal chelator. However, whether LA or DHLA binds transition metals in a redox-inactive is a question that hasn't been explored. We addressed this issue by evaluating the effects of LA or DHLA on transition metal-mediated oxidation. Results showed that only DHLA effectively lowered the redox activities of iron and copper (chapter 3). Based on these results, we tested whether DHLA removes metals from metalloenzymes, such as Cu-Zn superoxide dismutase or aconitase. Our findings indicate that DHLA does not chelate enzyme-bound metals, suggesting that it can only bind free non-protein bound iron (chapter 3).

To test the physiological relevance of above findings, we examined whether dietary feeding of LA (0.2% w/w, for 2 weeks) lowers age-related accumulation of iron in the brain. Our results showed that the administration of LA to old rats effectively reversed the age-related increase in iron, but this was not due to the direct

chelating activities of DHLA (chapter 4). The implications of these findings are profound in that we clearly demonstrated that chronic accumulation of iron in the aging brain is a reversible process. To our knowledge, no other compounds have been shown to be effective in normalizing chronic iron burden associated with the aging brain. Thus, LA may be a useful agent to be used as a tool to determine the potential underlying mechanisms responsible for the age-associated accumulation of iron.

As aging is a good model for oxidative stress, *per se*, we sought to determine the effects of LA on influencing various *in vivo* parameters relevant to age-related pro-oxidant balance. Using young and old rats as our model, we focused on understanding how mitochondrial decay influences the aging process and how treatment with “metabolic antioxidants”, such as LA, affects these age-associated changes.

Our findings indicate that mitochondria isolated from the hearts of aging rats display a heterogeneous patterns of decay; only interfibrillary mitochondria (IFM) show age-related increases in oxidant generation, declines in AA and thiol redox status, and increases in 4-hydroxynonenal modified proteins (chapter 6). Given the importance of IFM for supplying ATP required for cardiac muscle contraction and relaxation, the selective loss of IFM is likely to contribute significantly to the age-related loss in cardiac distensibility. Thus, our studies provide direct link between the age-associated impairment of cardiac function and mitochondrial decay.

The selective loss of IFM function and attendant increase in mitochondrial oxidant generation is likely to be a significant contributor to the heightened levels of oxidative stress observed in cardiac myocytes isolated from aging rats. Aging rat heart

exhibit significantly lower levels of tissue ascorbate and higher steady-state levels of 8-oxo-2'-deoxyguanosine (oxo<sup>8</sup>dG). Remarkably, a two-week dietary intervention with LA (0.2% w/w) to old rats significantly attenuated the increase in the rate of oxidant appearance in cardiac myocytes (chapter 5). Moreover, the significant age-related declines in ascorbate and glutathione redox status in the aging heart returned to levels seen in young animals. LA supplementation also lowered the age-related accumulation of 8-oxo-2'-deoxyguanosine (oxo<sup>8</sup>dG) in cardiac myocytes. The reversal in oxo<sup>8</sup>dG levels by LA suggests that the DNA repair capacity remains intact in the aging heart. These results suggest that treating old rats with LA can partially overcome the pro-oxidant effects of IFM decay in the aging rat heart.

Based on the benefits associated with LA treatment, it would be expected that cells should accumulate LA as part of its antioxidant defenses. Quite the contrary, the levels of "free" LA/DHLA are not detectable in non-supplemented animals [42]. Even when exogenously supplied, the rapid systemic clearance of LA (90% excreted in urine within 24 hrs) limits the steady-state levels of non-protein bound LA/DHLA in tissues [230]. These studies suggest that inherent mechanisms are in place to limit the intracellular levels of LA.

To determine whether LA-mediated improvements in tissue antioxidant status was due to direct effects of DHLA or was due to other factors, we further examined whether LA treatment can directly influence the expression of GSH synthesizing enzymes. Results showed that an acute administration of LA (40 mg/kg bw) for 48 hrs significantly reversed the age-dependent loss in the expression of  $\gamma$ -glutamylcysteine

ligase ( $\gamma$ -GCL) and restored the steady-state levels of hepatic GSH (chapter 7). The expression of  $\gamma$ -GCL and other phase II detoxification enzymes are under transcriptional control of NF-E2 related factor 2 (Nrf2). Interestingly, the basal nuclear level of Nrf2 declines significantly with age. Remarkably, acute treatment with LA attenuated the age-associated loss of Nrf2-dependent gene expression and reversed the loss of  $\gamma$ -GCL enzyme levels and activity.

Interestingly, other dithiol compounds, such as sulforaphane and isothiocyanates have all been proposed to confer long term antioxidant protection in cells and in animals by triggering the expressions of ARE containing genes [232,244]. The prolonged improvement in cellular antioxidant defense elicited by minute quantities of Phase II inducers (often effective in  $\mu$ M range) show their appeal to be used not only in the context of chemoprevention cancer but potential a novel notion of finding a chemoprotectant against the aging process [244,245].

One potential explanation for this may be that LA is acting as a mild stressor. Using 3T3-L1 adipocytes, Moini and co-workers demonstrated that LA increased in glucose uptake by acting as a mild oxidant [246]. Similarly, others have reported that LA (10 - 100  $\mu$ M) induced the formation of mitochondrial permeability transition, a non-selective permeabilization of inner mitochondrial membrane [247]. Our observations from chapter 8 suggest that the improvement in antioxidant defense capacity seen in LA treated old rats may be a reflection of cellular adaptive response to mild stress introduced by LA. The notion of LA acting as hormetic agent is not a unique property. Previous study by Wild and co-workers demonstrated that pyrrolidine

dithiocarbamate (PDTC) potently induced cellular expression of  $\gamma$ -GCL by increasing the intracellular concentration of copper [209]. Thus, the sum of our results appears to suggest a novel mechanism for the various anti-aging effects of LA. Further studies are required for elucidating the optimal dose and duration for maintaining optimal balance between toxicity and improved protection.

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## Appendix

### List of Publications

**Suh JH**, Zhu B, and Frei, B. (2003) Ascorbate does not act as a pro-oxidant towards lipids and proteins in human plasma exposed to redox-active transition metal ions and hydrogen peroxide. *Free Radical Biol Med.* **34**, 1306-1314.

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