

AN ABSTRACT OF THE DISSERTATION OF

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Title: Age-related Decrease in Resilience Against Acute Redox Cycling Agents: Critical Role of Declining GSH-dependent Detoxification Capacity

Abstract approved:

Tory M. Hagen

Over the past century, life expectancy in the United States has dramatically increased leading to an increasingly aging population with people reaching, and spending more years in 'old age'. While this unprecedented shift in population demographics represents great strides for humanity, it is not without cost. One consequence of longer life is the increased accrual of age-associated diseases and chronic pathophysiological conditions. This is evident in the fact that over 80% of Americans over the age of 65 have at least one chronic medical condition [275]. Thus, lifespan has outpaced 'healthspan', or the time of one's life spent free from disease and disuse syndromes.

The work in this dissertation is defined by the investigation of “health assurance” biochemical pathways, the failure of which lead to heightened risk for age-related diseases. In particular, I have focused on why resiliency to oxidative stresses decline significantly with age. This focus has led to a research project that ultimately pinpoints a loss in glutathione-dependent defenses as an underlying aging factor, which could enhance risk for a variety of age-related diseases.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a major transcriptional regulator of numerous anti-oxidant, anti-inflammatory, and metabolic genes. We observed that, paradoxically, Nrf2 protein levels decline in the livers of aged rats despite the inflammatory environment evident in that organ. To examine the cause(s) of this loss, we investigated the age-related changes in Nrf2 protein homeostasis and activation in cultured hepatocytes from young (4–6 months) and old (24–28 months) Fischer 344 rats. While no significant age-dependent change in Nrf2 mRNA levels was observed, Nrf2 protein content was attenuated by ~40% with age ($p < 0.05$, $N=4$). Treatment with anethole trithione (A3T), along with bortezomib to inhibit degradation of existing protein, caused Nrf2 to accumulate significantly in cells from young animals ($p < 0.05$), but not old, indicating a lack of new Nrf2 synthesis. We hypothesized that the loss of Nrf2 protein synthesis with age may partly stem from an age-related increase in microRNA inhibition of Nrf2 translation. miRNA-146a, increases by > 2-fold with

age and is predicted to bind Nrf2 mRNA. Transfection of hepatocytes from young rats with a miRNA-146a mimic caused a 55% attenuation of Nrf2 translation that paralleled the age-related loss of Nrf2. Overall, these results provide novel insights for the age-related decline in Nrf2 and identify new targets to maintain Nrf2-dependent detoxification with age. Having established that this major transcriptional regulator is attenuated with age, we next examined what effects this would have on toxicological resilience.

Isolated hepatocytes from young and old rats were exposed to increasing concentrations of menadione, a vitamin K derivative and redox cycling agent, an LC₅₀ for each age group was established, and results showed a nearly 2-fold increase in susceptibility to menadione (LC₅₀ for young: 405 μM; LC₅₀ for old: 275 μM) with age. Examination of the known Nrf2-regulated pathways associated with menadione detoxification revealed, surprisingly, that NAD(P)H:quinone oxido-reductase 1 (NQO1) protein levels and activity were induced 9-fold and 4-fold with age, respectively (p=0.0019 and p=0.018; N=3), but glutathione peroxidase 4 (GPX4) declined by 70% (p=0.0043; N=3). These results indicate toxicity may stem from vulnerability to lipid peroxidation instead of inadequate reduction of menadione semi-quinone. GSH declined by a 3-fold greater margin in old versus young rat cells given 300 μM menadione (p<0.05 and p≤0.01 respectively; N=3), and providing GSH synthesis substrates (400 μM N-acetyl-cysteine) to hepatocytes from old before menadione resulted

in a >2-fold reduction in cell death, suggesting that the age-related increase in menadione susceptibility likely stems from attenuated GSH-dependent defenses. Additionally, young rat hepatocytes maintained ~30% of their GSH content which suggested the possibility that mitochondrial GSH preservation may be critical for cell survival.

In order to determine the role of mitochondrial GSH (mGSH) loss in increased susceptibility to xenobiotic insult with age, markers of mitochondrial function were measured in intact and digitonin permeabilized isolated hepatocytes from young and old rats under a redox cycling challenge (300 μ M menadione; \sim LC₅₀ for old). Preliminary results show that, while the rate of mGSH loss under menadione challenge was similar in both age groups, the difference in basal mGSH with age (68 vs 36 nmol GSH/mg protein, N=1) ultimately resulted in a 50% loss of mGSH in old rat hepatocytes versus only 28% in young within 10 minutes of exposure. Examination of mitochondrial membrane potential ($\Delta\psi_m$), which is acutely regulated by mGSH content, showed a distinct loss in basal mitochondrial membrane potential ($\Delta\psi_m$) (\sim 21%). Additionally, within five minutes of menadione treatment, $\Delta\psi_m$ was not markedly reduced in young but had collapsed and passed the threshold for mitochondrial permeability transition pore opening (\sim 100 mV) in old rat hepatocytes. Further characterization demonstrated that basal respiration and respiratory reserve capacity, indicators of cellular bioenergetic capacity, were both significantly

reduced upon menadione treatment in old rat hepatocytes (34% and 72% respectively, N=4, $p<0.05$) but not in young. These results suggested that the age-related difference in mitochondrial function under menadione challenge might stem from mGSH regulated electron transport chain (ETC) components. We therefore examined proton leak, complex I, and complex II contributions to mitochondrial oxygen consumption rates under menadione challenge in both age groups. Results showed no marked effect in young rat hepatocytes, while old rat hepatocytes demonstrated significant increases in proton leak and complex II contributions to oxygen consumption rate (4.2 and 3.6-fold respectively, N=4, $p<0.05$), along with a significant decline in complex I contribution (4.8-fold, N=4, $p<0.05$). This data clearly demonstrates an age-related increase in mitochondrial susceptibility to menadione challenge, particularly in complex I, and provide a plausible mechanism that links this vulnerability to age-related mGSH perturbations.

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Age-related Decrease in Resilience Against Acute Redox Cycling Agents: Critical
Role of Declining GSH-dependent Detoxification Capacity

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APPROVED:

Major Professor, representing Biochemistry and Biophysics

Chair of the Department of Biochemistry and Biophysics

Dean of the Graduate School

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

Nicholas Oliver Thomas, Author

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Chapter 3: Shay, K.P., and Thomas, N.O. performed and analyzed the western blot experiments. All other experiments were performed and analyzed by Thomas, N.O. Thomas, N.O., Shay, K.P., and Hagen, T.M. wrote the manuscript.

Chapter 4: Thomas, N.O. performed and analyzed all experiments. Thomas, N.O., Shay, K.P., and Hagen, T.M. wrote the manuscript.

TABLE OF CONTENTS

	<u>Page</u>
Chapter 1. Introduction	1
1.1 Aging Population	2
1.2 Glutathione	3
1.2.1 Cellular GSH homeostasis	4
1.2.2 Nrf2-dependent transcriptional regulation of γ -GCL and GSH Synthetase.....	6
1.2.3 GSH cellular compartmentalization	7
1.2.4 Chemical reactivity of GSH	9
1.2.5 GSH as a substrate for antioxidant enzymes.	12
1.2.6 Glutathione and detoxification enzymes.....	14
1.2.7 Age-related Loss of GSH	17
1.3 Dissertation Hypothesis and Specific Aims	18
Chapter 2. Age-related loss of hepatic Nrf2 protein homeostasis: Potential role for heightened expression of miR-146a	22
2.1 Abstract	23
2.2 Introduction	24
2.3 Materials and methods	27
2.3.1 Reagents	27
2.3.2 Animals	27
2.3.3 Hepatocyte isolation and cell culture	28
2.3.4 Transfections.....	29
2.3.5 Nrf2 half-life.....	29
2.3.6 ARE-gene induction and quantitative PCR.....	30
2.3.7 ARE activity.....	30
2.3.8 Lysate preparation and immunoblots.....	31
2.3.9 miRNA microarray	32
2.3.10 Statistical analysis	32
2.4 Results	32

TABLE OF CONTENTS (Continued)

	<u>Page</u>
2.4.1 Nrf2 protein declines with age in rat hepatocytes.....	33
2.4.2 Increasing Nrf2 levels remediates lost ARE gene expression.....	33
2.4.3 Aging compromises Nrf2 activation of ARE-mediated gene expression.....	34
2.4.4 MicroRNA 146a targets Nrf2 translation and is increased with age.....	36
2.5 Discussion	39
2.6 Figures	45
Chapter 3. Glutathione Maintenance Mitigates Age Related Susceptibility to Redox Cycling Agents	52
3.1 Abstract.....	53
3.2 Introduction	55
3.3 Materials and methods.....	57
3.3.1 Reagents.....	57
3.3.2 Animals	57
3.3.3 Hepatocyte Isolation	58
3.3.4 Cell and Tissue Lysates.....	58
3.3.5 Assessment of Menadione Toxicity	59
3.3.6 NQO1 Activity Assay	60
3.3.7 Malondialdehyde Quantification.....	60
3.3.8 Immunoblotting.....	61
3.3.9 Glutathione Analysis	62
3.3.10 Statistical Analysis.....	62
3.4 Results	63
3.4.1 Acute menadione challenge causes hepatocellular damage and loss of viability.	63
3.4.2 Increased vulnerability to menadione with age is not caused by a loss in NQO1 or increase in Cytochrome P450 Reductase (CPR).	64
3.4.3 Age-related decline in hepatic GSH and GPX4 contribute to menadione-mediated cytotoxicity.	65
3.4.4 Age-related menadione toxicity is reversed by GSH precursors.....	68
3.5 Discussion	68

TABLE OF CONTENTS (Continued)

	Page
3.6 Figures	74
Chapter 4. The Age-Related Loss of Mitochondrial Glutathione Exacerbates Menadione-Induced Inhibition of Complex I.....	80
4.1 Abstract	81
4.2 Introduction	83
4.3 Methods and Materials	86
4.3.1 Reagents	86
4.3.2 Animals	86
4.3.3 Hepatocyte Isolation	87
4.3.4 Analysis of Hepatocyte Mitochondrial Respiration.....	87
4.3.5 Digitonin fractionation.....	88
4.3.6 Analysis of Mitochondrial Membrane Potential in Permeabilized Hepatocytes.....	89
4.3.7 Glutathione Analysis	90
4.3.8 Statistical Analysis	91
4.4 Results	91
4.4.1 Acute Menadione Challenge Causes Rapid Mitochondrial Glutathione Loss.....	91
4.4.2 There is an Age-Related Collapse of Mitochondrial Membrane Potential Under Acute Menadione Challenge.....	92
4.4.3 Loss of Mitochondrial Basal Respiration and Respiratory Reserve Capacity Under Menadione Challenge is Exacerbated With Age	93
4.4.4 Acute Menadione Challenge Significantly Effects ETC Complexes I and II in Old Rat Hepatocytes.....	94
4.5 Discussion	95
4.6 Figures	101
Chapter 5. Conclusions.....	106

LIST OF FIGURES

Figure	Page
Figure 2.1. Nrf2 protein levels but not mRNA levels decline with age in rat liver.....	45
Figure 2.2. Nrf2 over-expression results in similar induction of ARE regulated genes with age	46
Figure 2.3. Stress response of the ARE is attenuated with age.....	47
Figure 2.4. Both Nrf2 synthesis and degradation decline with age.....	49
Figure 2.5. Increased miR-146a with age reduces Nrf2 protein levels.....	50
Figure 3.1. Acute menadione toxicity is enhanced with age.....	74
Figure 3.2. Menadione detoxification scheme	75
Figure 3.3. CPR protein levels decrease while NQO1 protein levels and activity increase with age.....	76
Figure 3.4. GSH loss during menadione treatment is accelerated with age.....	77
Figure 3.5. GPX4 protein levels are significantly reduced with age which correlates with an increased appearance of menadione-induced MDA.....	78
Figure 3.6. Pretreating cells with NAC prior to menadione exposure improves resistance.....	79
Figure 4.1. Menadione treatment causes rapid GSH loss in mitochondria from both young and old rat hepatocytes.....	101
Figure 4.2. Menadione (300 μ M) challenge results in the collapse of the mitochondrial membrane potential only in old rat hepatocytes.....	102
Figure 4.3. There is an age-related increase in menadione-induced loss of respiratory reserve capacity	104
Figure 4.4. Menadione treatment (300 μ M) causes marked age-related differences in oxygen consumption parameters	105

Age-related Decrease in Resilience Against Acute Redox Cycling Agents: Critical Role of Declining GSH-dependent Detoxification Capacity

Chapter 1

Introduction

1.1 Aging Population

Over the past century, life expectancy in the United States has dramatically increased leading to an increasingly aging population with people reaching, and spending more years in 'old age'. In the next forty years, the percentage of Americans over the age of 65 is projected to grow from approximately one in seven (15%), to nearly one in four (24%). In contrast to that, the percentage of those between the ages of 18 and 65, or the working-age population, and those under 18 years old, are projected to decline by 5% and 3% respectively [1]. This is an unprecedented shift in the age demographics of America, and is a general reflection of aging populations across the developed world. This so-called "age-wave" will undoubtedly bring dramatic changes to the economic and social fabric of this country. But nowhere will the advancing age demographic display itself more prominently than in the burden on public healthcare it will engender. This is because increasing life expectancy also brings along with it significant increases in healthcare costs associated with age-related chronic diseases. Age itself is the leading risk factor for the main chronic diseases (cardiovascular diseases, cancers, obstructive pulmonary disorders, kidney disease, neurodegenerative diseases, diabetes, and sarcopenia) that currently elevate morbidities and drive public healthcare outlays [2–17]. Public healthcare costs are projected to increase dramatically along with the aging of the population

[18]. Thus, lifespan has outpaced 'healthspan', or the time of one's life spent free from disease and disuse syndromes.

To compress the morbidities that limit health in older adults, it will be vital to assess the molecular and cellular factors that lead to disease burden with age. By doing so, it is envisioned that novel preventative strategies can be developed in order to lengthen healthspan by lowering the risk for overt diseases of aging. My work is defined by the investigation of "health assurance" biochemical pathways, the failure of which lead to heightened risk for age-related diseases. In particular, I have focused on why resiliency to oxidative stresses decline significantly with age. This focus has led to a research project that ultimately pinpoints a loss in glutathione-dependent defenses as an underlying aging factor, which could enhance risk for a variety of age-related diseases.

Based on this general outline, the following sections will provide a brief overview of the main roles where compromised GSH-dependent detoxification may impart greater susceptibility to cellular dysfunction: the biochemical mechanisms how glutathione mitigates oxidant and toxicant stresses.

1.2 Glutathione

Glutathione (GSH) is a phylogenetically conserved molecule that is nearly

ubiquitous throughout aerobic life, and arose at the same time oxygen became an appreciable part of the atmosphere [19–22]. Glutathione is a tripeptide consisting of γ -glutamate, cysteine, and glycine, and it represents the most abundant low molecular weight, aqueous, sulfur-containing compound present in most living cells. Cellular concentrations of GSH range from 0.1 - 10 mM. The liver typically contains the highest amounts of GSH in mammalian organs (5 - 10 mM), owing to the very high synthetic capacity associated with the liver's role in detoxification of xenobiotic compounds from the diet [23–29]. GSH accumulates to such a high degree because cellular peptidases cannot hydrolyze the unusual γ -glutamyl bond between the glutamate and cysteine moieties. Instead, GSH must be exported from the cell where it can be acted upon by γ -glutamyltransferase located on the outer leaflet of cell membranes [30–34].

1.2.1 Cellular GSH homeostasis

Because of its ancient origins, GSH is believed to be one of the founder “antioxidants”, and is part of an array of protecting molecules (vitamins E and C are others) that limit oxidative damage against insults which are part of living in a high oxygen environment. GSH can be synthesized in nearly all aerobic organisms and in virtually every mammalian cell [24,25,35,36]. Synthesis of GSH is performed by the consecutive action of two cytosolic enzymes, γ -Glutamylcysteine ligase (γ -GCL) and GSH synthetase (GS). Both enzymes are

ATP-dependent with γ -GCL representing the rate limiting reaction. γ -GCL can exist as just a catalytic subunit (GCLC), but it is much more catalytically efficient when associated with a modulatory protein (GCLM). GCLC knockout animals are not viable, due to embryonic lethality, but transgenic mice where *gclm* has been ablated, do survive to maturity. However, GCLM knockouts typically have only 10-20% of the GSH seen in the wildtype animals, and they are highly sensitive to oxidative stress [35,37–39]. Enzymatic studies on the catalytic subunit and holoenzyme have shown that interaction of GCLC with GCLM decreases the K_m for glutamate and ATP by over 3 and 5-fold, respectively, and the V_{max} increases by over 4-fold [37]. The K_{cat} for the enzyme is very fast, which facilitates rapid GSH synthesis [37].

While GSH synthesis occurs constitutively under normal physiological conditions, it can also be upregulated when cells are under oxidative stress [23,35,40,41]. The γ -GCL enzyme acts as a redox sensor through the formation of a reversible intermolecular disulfide bond during oxidizing conditions. This leads to a gain of function conformational change that increases enzyme specificity for glutamate binding. This disulfide bond can be reduced when sufficient quantities of GSH are present, thus reducing enzyme function back to basal levels. In this way, GSH can regulate its own synthesis whenever sufficient substrates are available through feedback inhibition [42]. Under prolonged oxidizing conditions in the cell, transcriptional upregulation of both the GCLC

and GCLM subunits occurs [43,44].

1.2.2 Nrf2-dependent transcriptional regulation of γ -GCL and GSH Synthetase

Both the catalytic and modulatory subunits of γ -GCL, as well as GSH synthetase, are transcriptionally regulated by Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2 [45–47]. Nrf2 is a member of the Cap 'n' Collar (CNC) family, and contains a basic-leucine zipper (bZIP) DNA binding motif. Nrf2 is ubiquitously and constitutively expressed in most tissues, especially those frequently exposed to xenobiotics and exogenous reactive oxygen species (ROS) such as the liver, lungs, and gastrointestinal system [48–52].

Upon translocation to the nucleus, Nrf2 forms a complex with other proteins, binds to AREs in the 5'-flanking regions of detoxification genes and recruits transcriptional machinery to enhance gene expression. Nrf2 also recruits several other proteins necessary for transcriptional activation, including chromatin remodelers, and histone acetylases, deacetylases, and methyltransferases [53–55]. In the case of both GCLC and GCLM, studies by our lab and others have shown that Nrf2 regulates both the basal and inducible expression of these genes [56,57]. Additionally, Nrf2 knockdowns show significant reductions in both mRNA transcripts and the over-expression of Nrf2 or cellular treatment with Nrf2 inducers such as lipoic acid (LA) or anetholetrithione (A3T) increase

transcription [58,59]. While no AREs for GS have been identified, recent experiments have identified that overexpressing Nrf2 induces GS promoter activity while ablation of the Nrf2 gene decreases GS promoter activity by 66% [36].

1.2.3 GSH cellular compartmentalization

Due to its high concentration, and redox active sulfhydryl group, GSH is involved in many physiological functions throughout the entire cell. These roles include detoxification (see sections 1.2.5-6), enzyme regulation, and regulation of cellular functions such as differentiation and apoptosis [35,36]. With such a wide-ranging influence on so many diverse cellular processes, it is no surprise that GSH is located in nearly every cellular compartment including the nucleus, cytosol, endoplasmic reticulum (ER), and mitochondria. More importantly, it is now apparent that GSH exists, sub-cellularly, as different pools, which aid in specific tasks of particular organelles.

The main cellular GSH fraction is located in the cytosol. There, it is critical in cytosolic redox balance through the regulation of protein thiol-disulfide exchange and as an electron donor for multiple detoxification enzymes [25,60]. The cytosolic GSH/GSSG redox balance is approximately 1:30 - 1:100 in mammalian cells but has been measured up to 1:3000 in some yeast models. As

such, GSH maintains cysteine thiolates in cytosolic proteins in a reduced and functionally active redox state. In contrast to the cytosol, GSH is typically highly oxidized (GSH/GSSG redox couple =1:1 to 1:3) in the ER [61]. The low redox ratio of GSH in the ER facilitates the formation of disulfide bonds, likely by maintaining the correct activity of protein disulfide isomerases (PDIs), which catalyze disulfide bond formation along with the ER flavoprotein Ero1 [62–64]. There is also evidence that nucleus contains a distinct fraction of mitochondria that is not in equilibrium with the main cytosolic GSH pool. Nuclear GSH plays an important regulatory role in DNA synthesis, chromosome consolidation, nuclear matrix organization, redox maintenance of the cysteine residues in zinc finger motifs, and of course, protecting DNA from oxidative stress[65–67].

Of particular importance to the research involved in this dissertation, mitochondria also contain a distinct GSH pool that is not directly linked to the cytosolic GSH fraction. Mitochondrial GSH (mGSH) levels typically reflects the concentrations evident in the cytosolic pool, 1-10 mM, and constitute approximately 15% of the total cellular GSH levels. However, no GSH synthesis occurs in mitochondria, which instead must be obtained from the cytosol. To date, both the 2-oxoglutarate carrier (OGC) and the dicarboxylate carrier have been identified as transporters for GSH [68–72]. However, it is currently controversial as to the extent that these are the sole means for GSH uptake into mitochondria [73–75]. Thus, the regulation and uptake of GSH is still relatively

unclear. Despite this, it is well established that there is little or no passive diffusion of GSH into or out of the mitochondria; rather, uptake into the matrix of mitochondria is dependent on generation of a proton motive force. When the membrane potential is experimentally collapsed, even in the presence of ATP, GSH is not transported [76,77]. Additionally, loss of membrane fluidity, as seen in alcohol-fed rats, decreases GSH transport but the effect can be reversed through the addition of A₂C, a fluidizing fatty acid [78].

1.2.4 Chemical reactivity of GSH

Regardless of its cellular locale, the ultimate chemical reactivity of GSH is conferred by the free sulfhydryl from the cysteinyl residue. Thiols are considered “soft nucleophiles” due to the relative polarizability of the sulfur atom, and as such react preferentially and rapidly with “soft electrophiles” such as the imidoquinone of acetaminophen [79]. Most reactive metabolites are electrophiles and the GSH tripeptide is the principle soft nucleophile in the cell [80]. While GSH has no more reactivity than cysteine, the carboxylates of the glutamyl and glycyl residues pose a steric hindrance on the holo-tripeptide which results in a heightened pK_a of ~9.4 *versus* thiolates general (pK_a ~8.2) [81]. This increases the protonation of the thiol group at physiological pH and reduces the reactivity. Thus, GSH is not a particularly strong terminator of ROS in its own right. However, despite this, GSH is considered a key antioxidant and

can react with both oxidants and reductants [79,80]. GSH is a good chelator of redox-active iron and copper, which would prevent conversion of superoxide to more deleterious free radicals. Also, GSH chelates heavy metals, such as Hg and Pb, which also limits their ability to induce oxidative stress and cellular damage [79,80,82].

In addition to metal chelation, GSH is a prominent player in maintaining the cellular reduction/oxidation (redox) environment of the cell. GSH (reduced form) forms a redox couple with glutathione disulfide (GSSG) with a reported Eh value between -200 and -320 mV depending on cell type and cell cycle status. This places the GSH/GSSG couple high in the pecking order of other redox pairs, which include NADPH/NADP⁺, thioredoxin-SH/thioredoxin-SS, and Cysteine/Cystine. Only, the NAD(P)H/NADP⁺ redox pair has a higher reducing power in biological systems, and the amount of reduced to oxidized GSH is often considered a surrogate for measuring NADPH/NADP⁺ [83–90]. Considering that GSH concentrations are in the millimolar range, the GSH/GSSG redox pair readily interacts with the other redox couples, and participates in reversible oxidation and reduction reactions that, by-and-large, keep critical cysteine thiols in a reduced and active state. Thus, maintaining a high GSH/GSSG ratio maintains cellular metabolic homeostasis. In fact, GSH is considered to be an important redox “rheostat” that markedly affects cell cycle regulation, signal transduction, and also serves as a regulator for apoptotic and necrotic processes

[83,84,86,88,90].

One important mechanism, through which the GSH/GSSG ratio regulates the processes listed above, is via protein glutathionylation, or the formation of protein-GSH mixed disulfides. Glutathionylation has been shown to be a critical modulator of cellular signaling cascades and enzyme function, the latter of which is particularly evident in mitochondria [91–93]. Mitochondrial glutaredoxin (Grx2) can catalyze the reversible glutathionylation of protein thiols on complexes I, III, and IV of the electron transport chain. Complex I appears to be particularly sensitive to glutathione-mediated redox regulation [93–99]. Glutathionylation via a thiol-disulfide exchange between a protein thiol and GSSG can operate as a thiol protectant under periods of oxidative stress, as well as a mechanism for signal transduction [7,100]. Deglutathionylation is achieved by a thiol-disulfide exchange between a glutathionylated protein and GSH. As such, GSH is a critical substrate both as an antioxidant, and for redox signaling.

As oxidants and oxidative damage directly affect the degree of reduced to oxidized GSH, the tripeptide is a fundamental sensor and signal transducer for cellular response to ROS and RNS. Oxidative and nitrosative stresses are fundamental in the GSH/GSSG redox status, and as such GSH is a fundamental element of the signaling cascade evoked by ROS and reactive nitrogen species (RNS) [34,67,82,101]. When cells are under oxidizing conditions, GSH tends to decline and/or GSSG increases. Maintenance of the GSH/GSSG couple is performed by various enzymatic activities including synthesis of GSH, reduction

of the GSSG disulfide to 2 GSH via GSSG reductase, and export and degradation of GSSG. If cellular maintenance is insufficient, GSH loss and disruption of the GSH/GSSG redox couple can lead to irreversible cell damage and death.

The redox environment is frequently altered in disease states and imbalances in redox status are also known to result in disease. Decreased GSH levels are known to be associated with many diseases including lung, liver, cardiovascular, and neurological diseases. Supplemental or pharmaceutical therapies for regulating and improving GSH status could offer exciting avenues in disease prevention or delay.

1.2.5 GSH as a substrate for antioxidant enzymes.

A hallmark of aging evident across mammalian species is an increased appearance of highly reactive oxygen (peroxides, hydroxyl radical) and nitrogen (peroxynitrite) species [102–105]. Typically, increases in ROS and RNS has been assigned to enhanced superoxide emanating from increased inefficiencies in mitochondrial electron transport, from elevated activities of NADPH-dependent oxidases, and from accumulation of lipofuscin and inclusion bodies. Superoxide is relatively inert but can be converted into more deleterious and long-lived ROS and RNS, which may interact with and oxidize critical cellular components: DNA, proteins, and lipids, which lead to losses of function at the cellular, organ, and

organismal level. A large body of research across many aging models from invertebrates like drosophila and *C. elegans* to higher mammals like the rhesus macaque and chimpanzees clearly indicates that increases in ROS and oxidative damage play a critical role in the etiology and progression of age-related diseases [103,104,106–110].

GSH plays an important role in cellular defenses against ROS and RNS by acting as a substrate for vital cellular defense enzymes. In particular, GSH strongly regulates the amounts of harmful peroxides by providing reducing equivalents for the glutathione peroxidases (GPxs), which are a ubiquitous set of enzymes that detoxify an array of peroxides that arise as by-products of normal metabolism or that result from exposure to certain xenobiotic compounds. The role of GSH as an anti-peroxidant is represented by the following basic reaction scheme:



Thus, it takes two GSH equivalents to provide the electrons necessary for each peroxide to be converted to a non-reactive alcohol. While this reaction could involve any compound containing a free thiolate, the high concentrations of GSH and the prevalence of enzymes such as the GPxs that have evolved to utilize it as a substrate make it the predominant player. There are currently 8 known human

GPx isoforms named simply Gpx 1-8. Five of these are seleno-dependent enzymes (GPx1-4, and 6) whereas three are not (GPx5, and 7). High levels of Gpx activity are generally found in tissues that are exposed to high levels of oxygen such as the lungs and in red blood cells. However, activity is also high in tissues such as the liver and kidneys [39,111–115]. In particular, mitochondria contain both GPx1 and 4, the latter of which serves a vital role in preventing lipid peroxidation of the inner mitochondrial membrane [112,116–118]. The different isoforms of GPx have varying tissue distributions as well as substrate specificities. In most cases, excepting GPx7 and 8 which lack GSH binding sites, GSH concentration is the most important determining factor in enzymatic rate and the Michaelis constants (K_m values) are in the micromolar range, making them excellent peroxide terminators. Of particular note for this dissertation, one particular GPx isoform (GPx4), is unique in that it associates with cellular membranes and detoxifies harmful phospholipid hydroperoxides and cholesterol hydroperoxides; therefore, GSH and GPx4 comprise one of the chief protectant systems against xenobiotics that exert their toxicity through lipid peroxidation and resultant cell death pathways that result from compromised membrane integrity.

1.2.6 Glutathione and detoxification enzymes

Detoxification is the biological process that cells use to neutralize and/or

remove reactive or pro-reactive compounds, which are introduced from endogenous or exogenous sources and would normally damage or inhibit biological processes. In addition to the endogenous oxidant load, humans are constantly exposed to exogenous environmental challenges (drugs and compounds) that may cause toxicity. Many of these compounds, termed xenobiotics, are lipophilic which requires conversion to a water-soluble form in order to facilitate detoxification and elimination. These processes are catalyzed by phase I and II detoxification enzymes [119–121]. Phase I enzymes, which include the cytochrome P450 family, typically oxidize, reduce, or hydrolyze a compound in order to facilitate conjugation by phase II enzymes and antioxidants. Conjugation reactions include glutathionylation, glucuronidation, sulfation, methylation, and acetylation as well as others. Detoxification occurs throughout the body, but the majority of the reactions are located in tissues that act as barriers of entry for exogenous compounds. These tissues include the eyes, nose, mouth, skin, lungs, gastrointestinal tract and perhaps to the largest extent and most importantly to this work, the liver.

Glutathione-S-Transferases (GSTs) are a particularly important family of phase II detoxification enzymes that catalyze the conjugation of GSH with many potent electrophiles, metabolites and xenobiotics. These GSH-conjugates are then converted to mercapturic acids and excreted [122,123]. Through this mechanism, GSTs perform a critical role in the metabolism, detoxification, and

elimination of both endogenous and exogenous compounds including peroxides, drugs, and pesticides. This process prevents these compounds from potentially binding to proteins, DNA, or other cellular molecules and causing oxidative damage. There are three major families of GSTs: cytosolic, mitochondrial, and microsomal [124]. GST enzymes form homo and heterodimers of 25 kDa subunits. GST monomer subunits are organized into 10 classes with Greek letter designations.

There are seven mammalian classes of cytosolic GSTs: alpha, mu, omega, pi, sigma, theta, and zeta. They are ubiquitously expressed with liver, kidneys, intestine, adrenal glands, and testis showing the highest activity. In addition to catalyzing conjugation reactions for compound elimination, cytosolic GSTs can play a role in intracellular transport by binding hydrophobic ligands such as hormones [34,125,126]. Many of the cytosolic GSTs have overlapping function and substrate specificity, which is unsurprising in a critical and redundant detoxification system. Additionally, this overlap could serve to mitigate the propensity toward genetic polymorphisms in human cytosolic GSTs [124]. The effectiveness of glutathione conjugation depends of a several factors, but the first and most important is the sufficient cellular content and production of glutathione.

1.2.7 Age-related Loss of GSH

It is well established that aging animals have an increased susceptibility to toxins. Additionally, in humans, many drugs are tolerated at a lower threshold in the elderly than in the young [127–129]. GSH loss with aging has been demonstrated in many species, and is associated with an increased risk for age-related diseases such as chronic renal failure, diabetes, Alzheimer's and Parkinson's diseases, and atherosclerosis [102,127,130–132]. Maintenance of higher GSH concentrations is directly correlated to good health with increasing age, and people with chronic age-related diseases have lower mean levels of plasma GSH than healthy age-matched individuals [39,56,128,130,133,134].

The rate of GSH decline is often linked to what is known as an organism's intrinsic rate-of-aging. In humans, GSH levels remain stable for the first 40-45 years of life followed by a linear decline in GSH levels and a shift in the GSH/GSSG couple toward the oxidized state [102,135,136]. Much research has been devoted to identifying the mechanisms of GSH loss with aging. Our lab and others have investigated age-related changes in the mechanisms of GSH regulation extensively. We previously showed that in rodent tissues, ~30% of total GSH and as much as 50% of mGSH is lost with age [56,57]. Furthermore, we found that this loss can largely be attributed to age-related declines in the

activity of both the catalytic and modulatory subunits of γ -GCL as well as GSH synthetase.

Our group further identified that the age-related loss of GSH synthetic capacity is causally linked to an age-related decline in their transcriptional activator, Nrf2 [56,57]. The identification of Nrf2's role in GSH loss with age is an important discovery as to why GSH and GSH-dependent stress response mechanisms decline with age as Nrf2 regulates both the basal and inducible expression GSH synthesis enzymes as well as many, if not all, of the GSH-dependent detoxification enzymes such as the GSTs, GPxs, and GRxs. Thus, age-related vulnerability to both chronic diseases and acute toxicological challenge may result from a loss in GSH synthesis enzyme transcription and lower basal levels of GSH in cells and plasma.

1.3 Dissertation Hypothesis and Specific Aims

In the elderly, there is an age-related attenuation of many phase II detoxification pathways [120,137,138]. The Hagen lab was the first to identify that the major transcriptional regulator of many of these pathways, Nrf2, is reduced by as much as 50% in the aging rat liver and it has since been shown that this loss is evident in humans as well [56,59,106,120,139]. Additionally, while the mechanism of Nrf2 loss has not been fully explored, it results in a concomitant decline in the

synthesis and basal level of the important antioxidant and detoxification substrate glutathione, though intracellular concentrations can still be in the millimolar range. Despite ample literature reports on the importance of GSH in detoxification, cell signaling, maintenance of cellular redox balance, regulation of apoptosis, and a multitude of pathophysiologies, *whether this basal decline increases vulnerability to acute toxicological stressors, to what extent, and whether it can be remediated has not been thoroughly investigated.* This gap in knowledge represents a critical lack of understanding of the role of one of the most important small molecular weight antioxidant and detoxification substrates in age-related decline in detoxification capacity and prevents the potential development of remediation strategies. Thus, identifying the underlying causes of GSH loss with age and the consequences for resilience against acute toxicological challenge is necessary for identifying preventative and/or protective therapies.

To fill this gap, my work investigates the mechanism of why protein homeostasis of the transcriptional regulator of GSH synthesis enzymes, Nrf2, declines with age, the consequences of the basal GSH loss for cellular resilience against acute toxicological challenge, and the mechanism through which GSH loss increases cellular vulnerability. My project is based on two hypotheses:

- i) Age-related GSH loss is regulated by loss of Nrf2 protein homeostasis.

In order to test this hypothesis, we explore two questions:

- a. Why is there a decline in Nrf2 protein with age when a pro-oxidant environment in cells would suggest the opposite? We will answer this question using isolated hepatocytes from young (4-6 months) and old (24-26 months) Fischer 344 rats. This model recapitulates the aging phenotype of Nrf2 loss and can be used to identify potential lesions in Nrf2 regulation.
 - b. If Nrf2 levels are remediated, can the GSH synthesis genes (*Gclc/Gclm*) it transcriptionally regulates also be restored? This question will be addressed by overexpressing Nrf2 in isolated hepatocytes from young and old F344 rats for 24 hours before assaying *Gclc/Gclm* via qPCR.
- ii) The age-related decline in basal GSH pools will increase vulnerability to acute redox cycling compounds. This hypothesis advances several key questions:
- a. Is there an age-related increase in toxicity of an acute redox cycling compound that is detoxified in a GSH-dependent manner? We will address this question by acutely challenging isolated

hepatocytes from young and old F344 rats with increasing concentrations of menadione, a redox cycling compound that is detoxified through several GSH-dependent mechanisms, and evaluating cellular viability and the capacity relevant detoxification pathways (Cytochrome P450 (CYP), NqO1, GSH, GPx4).

- b. Does maintaining GSH levels in cells from old animals preserve detoxification capacity against acute redox cycling challenge? In order to assess the importance of GSH-dependent detoxification, we will pretreat old rat hepatocytes with N-acetyl-cysteine (NAC), a GSH synthesis precursor, and then reevaluate cell viability under acute menadione challenge.
- c. What role, if any, does age-related loss of mitochondrial GSH play in age-related loss of detoxification capacity? We will explore this question by challenging young and old rat hepatocytes to an acute menadione challenge and evaluating the effects on mitochondrial function (membrane potential, basal respiration, respiratory reserve capacity, mGSH, flux through complexes I and II of the ETC) in each age group.

Chapter 2

Age-related loss of hepatic Nrf2 protein homeostasis: Potential role for
heightened expression of miR-146a

Eric J. Smith, Kate P. Shay, Nicholas O. Thomas, Judy A. Butler, Liam F. Finlay,
Tory M. Hagen

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2.1 Abstract

Nrf2 regulates the expression of numerous anti-oxidant, anti-inflammatory, and metabolic genes. We observed that, paradoxically, Nrf2 protein levels decline in the livers of aged rats despite the inflammatory environment evident in that organ. To examine the cause(s) of this loss, we investigated the age-related changes in Nrf2 protein homeostasis and activation in cultured hepatocytes from young (4–6 months) and old (24–28 months) Fischer 344 rats. While no age-dependent change in Nrf2 mRNA levels was observed, Nrf2 protein content, and the basal and anetholetrithione (A3T)-induced expression of Nrf2-dependent genes were attenuated with age. Conversely, overexpression of Nrf2 in cells from old animals reinstated gene induction. Treatment with A3T, along with bortezomib to inhibit degradation of existing protein, caused Nrf2 to accumulate significantly in cells from young animals ($p < 0.05$), but not old, indicating a lack of new Nrf2 synthesis. We hypothesized that the loss of Nrf2 protein synthesis with age may partly stem from an age-related increase in microRNA inhibition of Nrf2 translation. Microarray analysis revealed that six microRNAs significantly increase > 2 -fold with age ($p < 0.05$). One of these, miRNA-146a, is predicted to bind Nrf2 mRNA. Transfection of hepatocytes from young rats with a miRNA-146a mimic caused a 55% attenuation of Nrf2 translation that paralleled the age-related loss of Nrf2. Overall, these results provide novel insights for the age-

related decline in Nrf2 and identify new targets to maintain Nrf2-dependent detoxification with age.

2.2 Introduction

The transcription factor Nuclear Factor, Erythroid Derived 2, Like 2 (NFE2L2 or Nrf2) is a gatekeeper of stress resistance without which cells succumb to both exogenous and endogenous toxins. Over 200 genes responsible for protein homeostasis, antioxidants, and detoxification are under the control of Nrf2 through the Antioxidant Response Element (ARE) binding site. While Nrf2 knockout mice are viable, they display a dramatically lowered ability to detoxify potential carcinogens [140]. Nrf2 is vital for protection against the molecular damage that leads to neoplasia, a risk that increases with age. Unfortunately, Nrf2 protein levels do not appear to be maintained during aging. In fact, we showed previously that Nrf2 levels decline in the aging rat liver [56], which reveals a problematic age-related deficit in the very organ that is responsible for detoxification.

In addition to this loss of detoxification capacity, aging is also characterized by an increase in endogenous reactive oxygen and nitrogen species, and a loss of endogenous antioxidant production [141–143]. It is now recognized that this pro-oxidant cellular environment, which is characterized by chronic inflammation, may be an intrinsic part of the aging process. This so-called

“inflammaging” contributes to a heightened risk for age-associated diseases (e.g. atherosclerosis, cancer, and dementias) and disuse syndromes (e.g. sarcopenia and frailty) [144]. As Nrf2 plays a key role in coordinating the cellular response to numerous pro-inflammatory insults, electrophilic xenobiotics, and metabolic perturbations, its decline during aging only exacerbates the already heightened risk for chronic pathophysiologies. However, we currently do not know why Nrf2 steady-state levels are lowered with age at the time of life when the need for detoxification is effectively increasing, nor do we know whether its induction with age is compromised.

Nrf2 homeostasis is regulated by an array of interconnected transcriptional, translational, and post-translational mechanisms that allow it to fine-tune gene expression to an array of divergent stress stimuli [52]. Under quiescent conditions, this basic leucine zipper, cap-“N”-collar transcription factor is normally maintained at very low steady-state levels [145]. The basal content of Nrf2 is sufficient for its binding to cis-acting AREs located in the 5'-flanking regions of target antioxidant and ARE-containing genes. Under acute pro-oxidant conditions or in the presence of electrophilic compounds, Nrf2 rapidly accumulates in the nucleus and enhances expression of detoxification genes. The accumulation of Nrf2 is due in part to its dissociation from Keap1, a Cul3 ubiquitin ligase adaptor protein, thus attenuating its proteasomal degradation. However, this mechanism is only able to preserve existing Nrf2 protein, which is

present at very low levels. This seemingly contradictory phenomenon has been explained by several reports showing that increased translation is a critical means to quickly adjust Nrf2 protein levels for adequate cellular response [58,146,147]. For example, Nrf2 mRNA contains an internal ribosomal entry site (IRES), which allows its translation even during a catabolic state when global cap-dependent translation is reduced [148].

An additional layer of translational control over Nrf2 mRNA may be mediated by microRNAs (miRNAs), a class of small RNAs derived from distinctive hairpin transcripts that are ubiquitous across the plant and animal kingdoms. Once processed, miRNAs average approximately 21–22 nucleotides in length and associate with Argonaute proteins as part of an RNA-induced silencing complex. These complexes primarily serve to control gene expression through post-transcriptional repression of translation. Several miRNAs can regulate the translation of Nrf2 mRNA and therefore lower its steady state levels [149,150]. However, it has never been shown that miRNAs are responsible for the age-related loss of Nrf2.

Despite the importance of Nrf2-mediated response with age, and the complexity of Nrf2 proteostasis, there have been few studies to date that have examined the potential mechanism (s) involved. In this study, we sought to elucidate the mechanism (s) that results in the age-related loss of Nrf2 protein levels using

hepatocytes from old and young rats. We hypothesized that Nrf2 protein synthesis declines with age, resulting in the loss of basal Nrf2 protein levels and stress response. Herein, we also describe the contribution of an miRNA, miR-146a, that lowers Nrf2 translation.

2.3 Materials and methods

2.3.1 Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Collagenase type IV was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Anti-Nrf2 (sc-13032) and anti-Keap1 (sc-15246) were ordered from Santa Cruz Biotechnology (Santa Cruz, CA). Bortezomib was obtained from Millennium Pharmaceuticals (Cambridge, MA). PVDF transfer membrane was purchased from Millipore (Billerica, MA). Dual Luciferase Assay Kit was purchased from Promega (Madison, WI).

2.3.2 Animals

Fischer 344 male rats, both young (4–6 months) and old (25–28 months), were purchased from the National Institute on Aging animal colonies. The rats were allowed to acclimatize in the Linus Pauling Institute animal facility for a

minimum of 1 week on a 12 h light cycle (7 am–7 pm) and fed standard chow ad libitum. All animal work was approved and in accordance to IACUC guidelines (Assurance number: A3229-01). The AAALAC-accredited Laboratory Animal Resources Center (LARC) provided management and veterinary care.

2.3.3 Hepatocyte isolation and cell culture

Hepatocyte isolation was performed as described previously [151]. Briefly, the liver was perfused with Hank's balanced salt solution, and disassociated to a single cell suspension using a collagenase solution (1 mg/ml). The resultant cell suspension was filtered through sterile gauze to remove connective tissue and debris. Parenchymal cells were isolated using gravity filtration and washed four times with Krebs–Henseleit solution, pH 7.3. Cell count and viability were assessed using trypan blue exclusion. Freshly isolated hepatocytes were suspended in a modified Williams' E media (5% FBS, 1 mM dexamethasone, 100 ng/ml insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin), dispensed onto collagen coated cell culture plates and incubated for 16 h at 37 °C in 5% CO₂ atmosphere before being used in experiments. Hepatocytes were seeded at 2.5×10^5 cells/ml of media for regular cell culture, or 1.25×10^5 cells/ml of media for culture transfection. We have demonstrated that these outlined culture conditions maintain the age profile of hepatocytes with respect to Nrf2 for at least three days in culture [120].

2.3.4 Transfections

Plasmid transfections of hepatocytes were achieved with the jetPEI-hepatocyte transfection reagent (Polyplus-transfection SA, Illkirch, France). Hepatocytes were incubated with 2 µg plasmid and the recommended amount of jetPEI after hepatocytes had been in culture for 30 h. Transfection efficiency was assessed to be ≥60% by parallel transfection of EGFP vector and cell counting. miRNA transfection of hepatocytes were achieved with mirVana miRNA mimic (Ambion) using RNAiMax (Life Technologies) following the manufacturer's protocol. The miRNA levels were measured using the Taqman MicroRNA Assay (Life Technologies).

2.3.5 Nrf2 half-life

Hepatocytes were transfected with a human influenza hemagglutinin-tagged Nrf2 (isoform BC061724) expression vector (pHA-Nrf2) with jetPEI as detailed above. After 20 h, hepatocytes were treated with 100 µM cycloheximide. Hepatocytes were harvested every 20 min and the proteins were isolated as described. Immunoblots were used to quantify Nrf2 levels. Statistical analysis was done by a two-way ANOVA.

2.3.6 ARE-gene induction and quantitative PCR

Hepatocytes were transfected with a human influenza hemagglutinin tagged Nrf2 (isoform BC061724) over-expression vector (pHA-Nrf2) or empty vector with jetPEI as detailed above. Cells were harvested 16 h after transfection, total RNA was collected from hepatocytes with Trizol (Life Technologies, Carlsbad, CA), and reverse transcription was performed using the Retroscript Kit (Life Technologies) following the manufacturer's protocol. The PCR was done on a StepOnePlus PCR machine (Life Technologies) using Taqman Universal Mastermix. Relative quantities were calculated based on $\Delta\Delta C_t$ between cells transfected with pHA-Nrf2 and empty vector and assuming an amplification efficiency of 2. All primer-probe mixtures were purchased from Life Technologies except for Nrf2, which were ordered from Eurofins MWG Operon (Huntsville, Alabama) with the following sequence and modifications: TTTTCCAGTGAGGGGATCGATGAG, GTCAGCTACTCCCAGGTTGCCCA, and [6-FAM]ACCACTGTCCCCAGCCCAGAGGCCAC [BHQ1aQ]. Quantification was normalized to the housekeeping gene EIF2A.

2.3.7 ARE activity

Hepatocytes in culture were transfected with 1.95 μg ARE-Luc, a Gclc-promoter driven PGL4 luciferase vector, and 0.05 μg of a Renilla expression vector (pRL-

CMV). Cells were incubated 18 h after transfection and then treated with either vehicle control (DMF), 50 μ M 5-(4-methoxyphenyl)-3H-1,2-dithiole-3-thione (A3T), or 100 μ M R- α -Lipoic Acid (LA) (Makwood). After 16 h cells were harvested and assayed for luciferase activity using the protocol provided with the Dual Luciferase Assay Kit (Promega).

2.3.8 Lysate preparation and immunoblots

Cultured hepatocytes were washed twice with PBS, pH 7.4, and then collected by scraping. Nuclear and cytoplasmic protein extracts were collected by the use of Sigma-Aldrich CellLytic NuCLEAR isolation kit following the manufacturer's protocol. Protein samples were denatured by addition of 2X Laemmli loading buffer and heating at 90 °C for 5 min. Protein separation was performed using a neutral Bis-Tris acrylamide gel and following the BioRad XT Bis-Tris PAGE XT-Mes protocol. Transfer was accomplished using the Bjerrum Schafer-Nielsen transfer buffer with 20% methanol on a semi-dry transfer apparatus. Proteins were deposited on PVDF transfer membrane. All blots were blocked with a 5% milk (w/v) TBS-T solution overnight at 5 °C. Primary and secondary antibodies were each incubated on the membranes for 2 h. Densitometry, performed with the ImageJ software package ([http:// imagej.nih.gov/ij/](http://imagej.nih.gov/ij/)), was used for graphical representation and to perform statistical analysis.

2.3.9 miRNA microarray

Liver tissue from young and old rats was collected and miRNAs isolated using the PureLink miRNA Isolation Kit (Life Technologies). Samples were frozen in liquid nitrogen and shipped to LC Sciences (Houston, TX) for analysis. Microarray analysis covered the entire *Rattus norvegicus* miRBase set (miRBase release 12.0). Background subtraction, normalization, and statistical analysis were provided by LC Sciences. Significance was calculated by ANOVA and corrected for multiple comparisons.

2.3.10 Statistical analysis

All statistical analysis was performed using Excel (Microsoft, Inc.) and Prism 7 (GraphPad Software, Inc.) For comparisons between two samples, two-sided Student's t-test was used. Differences between samples that resulted in a p-value of < 0.05 were considered statistically significant. Statistical analysis between multiple endpoints was analyzed by two-way ANOVA and multiple comparisons were evaluated by Tukey's post-hoc method.

2.4 Results

2.4.1 Nrf2 protein declines with age in rat hepatocytes

We previously showed that constitutive Nrf2 binding to a core consensus ARE sequence declines with age, indicating that basal expression of ARE-mediated genes may be compromised [151]. Fig. 1a and b shows a marked decline of Nrf2 protein with age; overall steady-state Nrf2 levels were $42 \pm 12\%$ lower in livers of 24-month old rats than in young controls ($p < 0.05$). Moreover, Nrf2 loss was coincident with a significant attenuation of expression of genes that are constitutively regulated by Nrf2. As shown in Fig. 1c, mRNA content of Gclc, Gclm, and Gst2a exhibited age-dependent declines of 19.6%, 31.4%, and 40%, respectively. Because Nrf2 mRNA levels vary in tissues [152] and in response to certain toxicological stressors [153–155], we sought to determine whether lowered Nrf2 transcript levels may be responsible for the age-related loss of Nrf2 protein content. On the contrary, hepatic Nrf2 mRNA values exhibited no significant age-dependent changes (Fig. 1c; $p = 0.14$), indicating that dysregulation of Nrf2 transcription with age does not account for attenuation of Nrf2 protein levels.

2.4.2 Increasing Nrf2 levels remediates lost ARE gene expression

To determine whether replenishing Nrf2 hepatic protein content may remediate the observed declines in ARE-dependent gene expression, we transfected

hepatocytes isolated from young and old rats with a CMV-driven pHA-Nrf2 expression vector. Under Nrf2 overexpression, the decline in ARE-associated target gene expression was reversed, where Gclc and Gclm mRNA content were at least 3-fold higher than observed in hepatocytes transfected with an empty vector (Fig. 2; $p < 0.05$), and there were no longer any age associated differences in their mRNA levels. These results show that the lower steady-state levels of Nrf2 primarily drive the observed loss of ARE-mediated detoxification gene expression with age.

2.4.3 Aging compromises Nrf2 activation of ARE-mediated gene expression

Our results point to a connection between lower Nrf2 steady state levels and decrements in stress response genes with age. However, it is not known whether Nrf2 levels are still sufficient to initiate a proper response to stress stimuli. To determine the extent of any age-dependent changes in Nrf2 stress response, we transfected hepatocytes from young and old rats with an ARE-containing luciferase reporter (ARE-luc), then treated the cells with known inducers of ARE-mediated Nrf2 gene expression. Treating cells from young animals with 100 μM R- α -lipoic acid (LA) [56] or 50 μM anethole trithione (5-[4-methoxyphenyl]-3H-1,2-dithiole-3-thione; A3T) [156], yielded a robust activation of the ARE-luc reporter vector, indicating a strong induction of the Nrf2-mediated stress resistance response in these cells. However, hepatocytes derived from old

animals showed only a small induction in luciferase activity with either LA or A3T, which was 6.5- and 7.0-fold lower, respectively, than what was observed in cells from young rats ($p < 0.05$) (Fig. 3). These results show that the age-related loss of Nrf2 not only compromises basal expression of certain ARE-mediated genes, but also has the potential for limiting the induction of ARE-mediated stress response from xenobiotic and stress stimuli.

Nrf2 steady-state levels are an interplay between its rates of synthesis and degradation. Considering that Fig. 1c shows no decrements in Nrf2 mRNA levels with age, we next examined the accumulation of the Nrf2 protein itself under stress stimuli in cells pretreated with bortezomib (BTZ), an inhibitor of the 26S proteasome, to prevent Nrf2 turnover. While Nrf2 levels accumulated in cells from young animals both basally and with LA or A3T stress induction, the same was not true in cells from old animals (Fig. 4A and B). In fact, no significant increase in Nrf2 occurred in cells from old animals, and the use of BTZ shows that this was not due to increased Nrf2 proteasomal degradation. In a follow-up experiment, we measured the levels of Keap1 by immunoblot. As Keap1 is a Cul3 ubiquitin ligase that facilitates Nrf2 degradation by the 26S proteasome, a significant increase in Keap1 in cells from old animals vs young would suggest that more Nrf2 degradation occurs with age. Contrary to this, we found that Keap1 levels declined 42% on an age basis (Fig. 4C) ($p < 0.05$). This surprising result prompted us to measure Nrf2 half-life in both sets of hepatocytes. We

overexpressed Nrf2 in hepatocytes from young and old rats and directly quantified the rate of Nrf2 turnover using cycloheximide as a means to block protein degradation. Results showed marked differences in Nrf2 turnover rates between young and old rat hepatocytes. A single-phase decay model to fit the data revealed that Nrf2 half-life increased >5-fold with age ($p < 0.05$; Fig. 4D), with $T_{1/2}$ estimates of 21.6 ± 3.0 and 119.8 ± 21.5 min in young and old hepatocytes, respectively. These results, along with the age-related loss of Keap1, suggest a markedly slower rate of Nrf2 degradation, showing that Nrf2 degradation is not responsible for the steady-state decline in hepatic Nrf2 levels with age, and may even be a mechanism used by the cell to compensate for Nrf2 loss. All of these results are in accordance with our previous data in immortalized cells [58] showing that Nrf2 mRNA translation is an important aspect of its ability to respond quickly to oxidative or xenobiotic stress, whereas it was formerly supposed that all Nrf2 entering the nucleus was being rescued from Keap1-mediated degradation. However, our current data suggests for the first time that the age-related loss of Nrf2 may be due to a change in its translation.

2.4.4 MicroRNA 146a targets Nrf2 translation and is increased with age

While Nrf2 translation is lower with age, the partial induction of Nrf2 with A3T shows that new synthesis of Nrf2 is still functional, albeit at a markedly lower

rate. Because of growing evidence that mRNA translation in general and Nrf2 translation in particular are fine-tuned to environmental conditions via short non-coding interfering RNA (miRNA) [150], we hypothesized that the sharp attenuation of hepatic Nrf2 mRNA with age stemmed from an increase in miRNA-dependent inhibition. Accordingly, a top-down analysis of age-dependent changes to microRNA was performed using tissue extracts from young and old rat liver. Of the microRNA analyzed, only thirteen transcripts met both the 2-fold statistical threshold for significance. Most of these miRNA species exhibited an age-associated decline (data not shown), which is in agreement with other reports showing dicer-dependent decline in miRNA maturation with age [157]. However, a small cadre of miRNA transcripts significantly increased on an age-related basis (Fig. 5a). While there are currently few reports identifying genes associated with many of these miRNAs, it does appear that most, if not all, of the miRNA species that become elevated with age (34a, 146a, 28, and 101a) are also known to be induced under chronic inflammatory conditions [158–161]. The oxidant-enriched milieu common in aging tissues has been hypothesized to contribute to a sterile chronic necro-inflammatory environment that goes unresolved by cellular stress response pathways [162,163].

Using mirSVR sequencing analysis and available literature reports, six miRNAs were identified [148,163–165] that increased significantly with age and/or

could theoretically bind to the 3' region of Nrf2 mRNA. A preliminary test was performed by transfecting chemically modified double-stranded RNAs that mimic these miRNAs into the rat hepatoma cell line H4IIE to determine their potential for inhibiting Nrf2 translation (data not shown). While the other miRNAs failed to inhibit Nrf2 expression in this immortalized cell line, the rno-miR-146a mimic (rno = *Rattus norvegicus*) markedly reduced Nrf2 protein and thus was selected for further experimentation. In order to confirm the array results, liver tissue from young and old rats was harvested, microRNA isolated, and RT-qPCR was performed (Fig. 5b). This analysis revealed a 2.7 ± 0.3 -fold increase in miR-146a in aging rat liver versus young controls, providing strong evidence this is an age-affected miRNA in the liver.

In order to investigate whether miR-146a influences the expression of Nrf2, hepatocytes from young rats were transfected with the mimic of the endogenous version, as used above in immortalized cells. Immunoblot analysis showed that Nrf2 protein levels were significantly lower in mimic-transfected cells versus those transfected with a scrambled control (Fig. 5c). As miRNAs either affect mRNA translation or bind and elicit message degradation, additional experiments examined whether rno-miR-146a altered Nrf2 mRNA levels and Nrf2-controlled genes (Fig. 5d). qPCR analysis revealed that rno-miR-146a treatment resulted in no significant difference in Nrf2 mRNA levels between the control and rno-miR-146a-treated samples. This is despite the observed change

in its protein accumulation, which suggests that rno-miR-146a inhibits ribosomal association of Nrf2 mRNA but does not induce its degradation. However, mRNA levels of the Nrf2-mediated genes, Gclc and Gclm, were lowered by the mimic treatment, $32.1 \pm 4.5\%$ and $13.4 \pm 2.6\%$ respectively. These data reflect the loss of Nrf2 protein synthesis in spite of preserved mRNA levels seen in liver tissues and isolated hepatocytes from old rats. Thus, the elevated level of miR-146a with age is consistent with a role in the attenuated translation of Nrf2.

2.5 Discussion

Nrf2 is not only involved in detoxification, but is increasingly recognized as an important longevity-assurance transcription factor because it regulates expression of numerous genes involved in stress resistance and also metabolism genes associated with longevity and health. Even some of the longevity enhancing effects of dietary restriction may stem from Nrf2-mediated gene regulation [166]. In accordance with our previous work [58] showing complex control of Nrf2 levels through mRNA translation, we found that the age-related lesion in Nrf2 proteostasis and inducibility is attenuated Nrf2 translation. Given the attenuated response to LA and A3T (Fig. 3), our data clearly point to a deficit in synthesis of Nrf2 protein with aging.

Decline in protein translation appears to be a trait of aging, and our data indicates that the decline in Nrf2 synthesis is in keeping with this general trait [167]. However, because Nrf2 has such a short half-life, Nrf2 levels and the genes that it regulates may be more adversely affected than other proteins with longer half-lives. We show that steady-state Nrf2 levels fall by approximately 40% with age despite a significantly slower turnover rate. Thus, adaptive mechanisms to maintain steady-state amounts of Nrf2 are not adequate as the aging process gathers pace.

The precise mechanism(s) that lower overall steady-state Nrf2 with age are yet to be completely defined. Our current data indicates that miRs, which arise during pro-inflammatory conditions, may be at least one cause for attenuated Nrf2 translation. We show that miR-146a reduces Nrf2 translation directly or indirectly due to its own increase with age, though it is likely one of several miRNAs with this influence. Herein, we showed that only miRNAs associated with inflammation increase with age in rat liver, while miRNA species connected to other metabolic pathways actually decline. miR-146a was the most abundant inflammation-induced miRNA that increased with age, and has a predicted binding sequence in the 3'-flanking region of Nrf2. Moreover, mimicking the age-related increase in miR-146a in hepatocytes from young animals partially recapitulates the loss of Nrf2 seen with age. While these results do not rise to the level of a cause-and-effect relationship, it does suggest the possibility that the

pro-inflammatory environment of the aging rat liver chronically induces expression of miR-146a, which in turn, adversely affects Nrf2 translation.

The loss of steady-state Nrf2 with age may be a compensatory mechanism for the increased risk of neoplasia. Although Nrf2 is responsible for xenobiotic detoxification and is therefore protective against potentially DNA-damaging compounds [50], the end result of Nrf2 activation is cytoprotection and, in many cases, escape from apoptosis [168]. Increased Nrf2 has been found in some cancers, and Nrf2 has also been implicated in cancer progression via its activation of metabolic enzymes in the pentose phosphate pathways [140]. Thus, lower Nrf2 at a basal level would not be detrimental as long as the system is still inducible. Unfortunately, this is not the case; when we induced Nrf2-regulated gene expression with A3T or LA, Nrf2-dependent stress response was severely attenuated compared to that in young animals (Figs. 3 and 4). While it remains to be fully elucidated the extent to which Nrf2 translation is activated under stress stimuli, these results do indicate that the attenuated Nrf2 translation of aging may be an underlying factor in the well-known age-dependent loss of xenobiotic and oxidant-induced stress response. In agreement with this, we previously showed that old animals are more vulnerable to exposure to lipid hydroperoxides, which are detoxified in a glutathione- and Nrf2-mediated manner [169]. Other studies have also demonstrated an age-related vulnerability to various drugs including H₂-receptor antagonists (antacids),

anesthetics, acetaminophen, and alcohol. All of these drugs are detoxified via Nrf2-dependent genes [170–172]. Thus, the age-related loss of Nrf2 translation may play a pivotal role in vulnerability to a variety of xenobiotic insults, further justifying the growing public health concerns associated with polypharmacy in the elderly [173,174]. As definitive examination of age-related changes in detoxification capacity, and its potential consequences on the therapeutic threshold of pharmacological agents in older individuals are scant, further work will be necessary to elucidate the extent and precise nature that the loss of Nrf2 protein homeostasis plays in increased susceptibility to environmental and pharmacological insults with age.

Outside of its involvement in susceptibility to acute toxicological insults, attenuated Nrf2 translation may be a factor in increasing the risk for chronic age-dependent pathophysiologies. For example, we previously demonstrated that significant age-related changes occur to hepatic gene expression where an ontological analysis of transcript changes revealed enrichment of immune response, immune cell infiltration, pro-inflammatory, and tissue remodeling genes [162]. Most importantly, there was no apparent coincident increase in expression of antioxidant/detoxification genes. Therefore, the loss of Nrf2 could directly contribute to perpetuation of the low-grade inflammation associated with aging. Nrf2 directly regulates numerous genes that act as negative feedback inhibitors to down-regulate cytokine and NF κ B-mediated inflammation, which

suggests that loss of Nrf2-mediated target genes may play a critical role for numerous pathophysiologies where chronic inflammation is part of the underlying etiology (e.g. dyslipidemia, fibrosis, cirrhosis, and cancer). Given the prevalence of miR-146a in inflammation, it is also interesting to note that the loss of Nrf2 translation may not only potentiate chronic inflammation, but may be perpetuated by the necro-inflammatory environment of the aging liver. The advantages of “cross-regulation” between inflammation and Nrf2, via miRNA, are presently unclear; however, it may be an example of antagonistic pleiotropy. Elevated miR-146a is a normal response to inflammation and appears to be a feedback mechanism to lower inflammatory response. Normally, inflammation is a transient condition and therefore so is the increased expression of miR-146a; however, in the aged animals inflammation persists. The age-related loss in Nrf2 translation may thus be an indirect consequence of persistent upregulation of pro-inflammatory cytokines. The resulting attenuation of Nrf2 would ironically limit the means to resolve the inflammatory response.

In summary, we have identified, for the first time, an age-related change that results in the attenuation of Nrf2 protein levels in the liver of old rats. Chiefly, Nrf2 protein synthesis declines with age. This observation can, in part, explain why Nrf2 levels decline despite the age-related increase in ROS. This results in the increased susceptibility to acute stressors that rely on basal levels of Nrf2 for detoxification and likely chronic challenges as well. Further research will be

required to characterize the consequence of the loss of Nrf2 translation in regards to chronic inflammatory conditions. Finally, restoration of Nrf2 synthesis, perhaps via disruption of miR-146a, and the effects of this reversal continue to be investigated.

Acknowledgments

This work was supported by NIH Grant P01AT002034 and NIH R01 2AG17141 and a grant from the Medical Research Foundation of Oregon (Grant number 2014-14-1837).

2.6 Figures

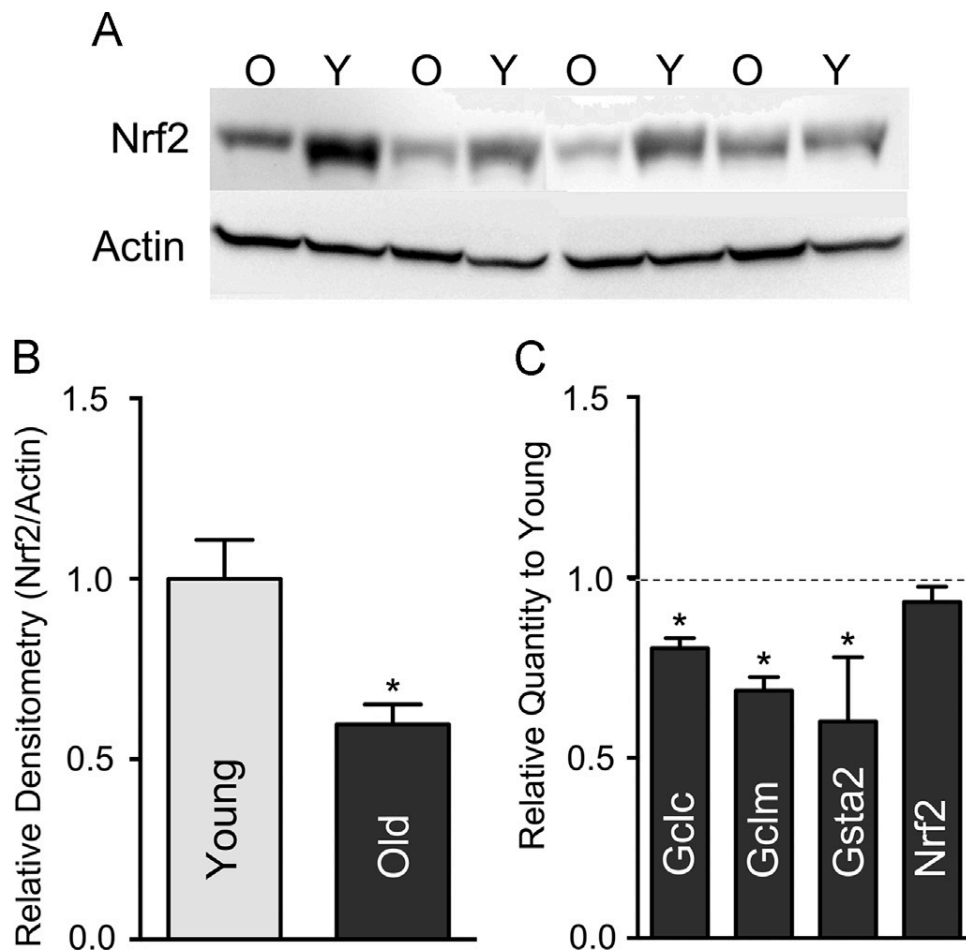


Figure 2.1. Nrf2 protein levels but not mRNA levels decline with age in rat liver. (A) Liver tissue lysate from young (Y) and old (O) rats were assayed for Nrf2 protein content by immunoblot. Actin was utilized as a loading control and housekeeping gene. (B) Densitometry graph for the expression of Nrf2 protein, N = 4; * $p < 0.05$. (C) QPCR was utilized to assay the relative expression of the Nrf2 regulated genes: gamma glutamyl cysteine ligase catalytic and modulatory subunits (Gclc and Gclm), and glutathione S transferase subunit a2 (Gsta2), as well as Nrf2 itself. Graph shows old relative to young (Y = 1.0). Messages that decline significantly with age are noted by * ($p < 0.05$; N = 7).

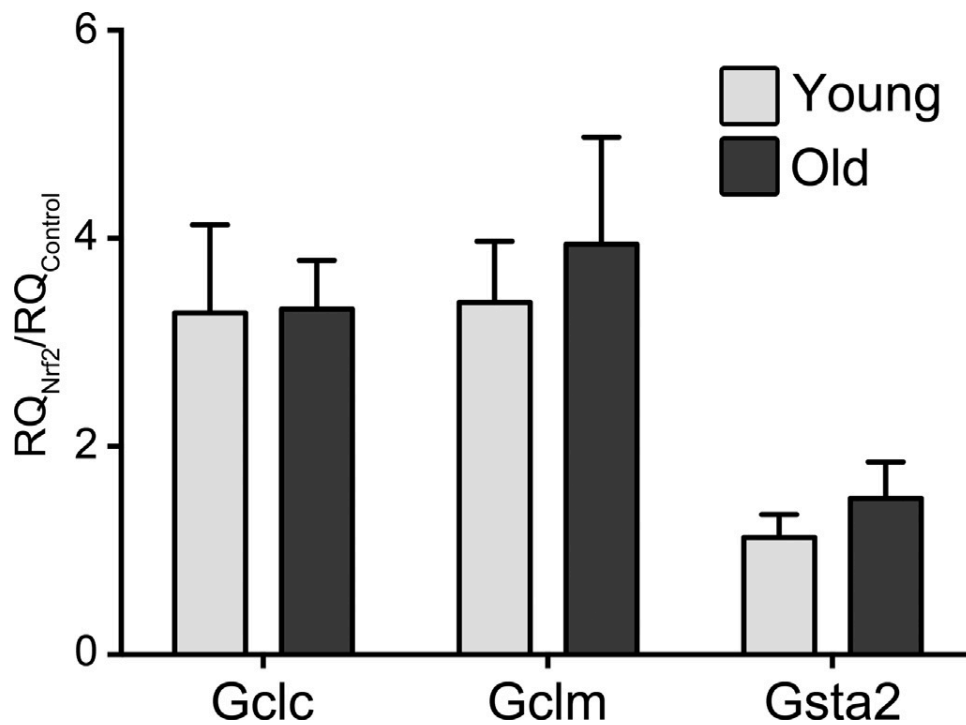


Figure 2.2. Nrf2 over-expression results in similar induction of ARE regulated genes with age. Cultured hepatocytes from young and old rats were transfected with pHA-Nrf2. After 24h, qPCR was utilized to assay the relative expression of genes regulated by Nrf2. Analysis shows that there is no significant difference between young and old samples ($p = 0.05$).

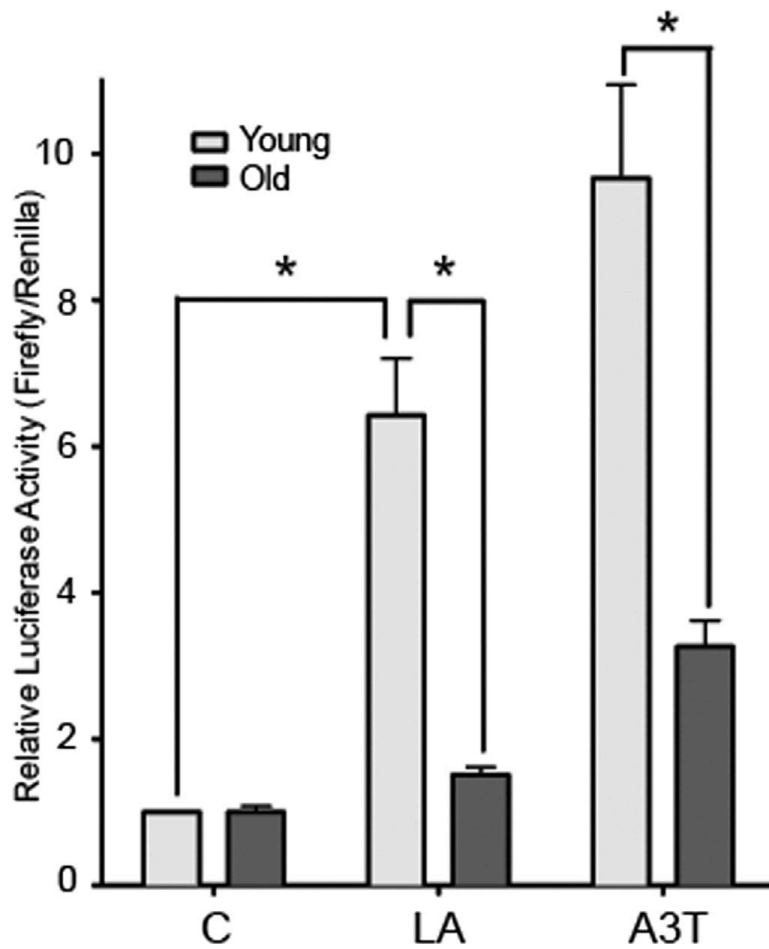
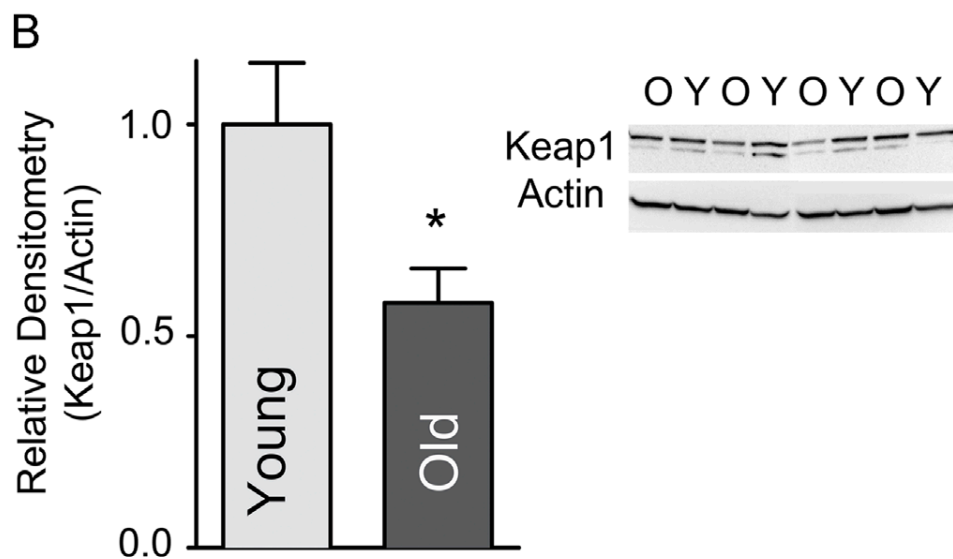
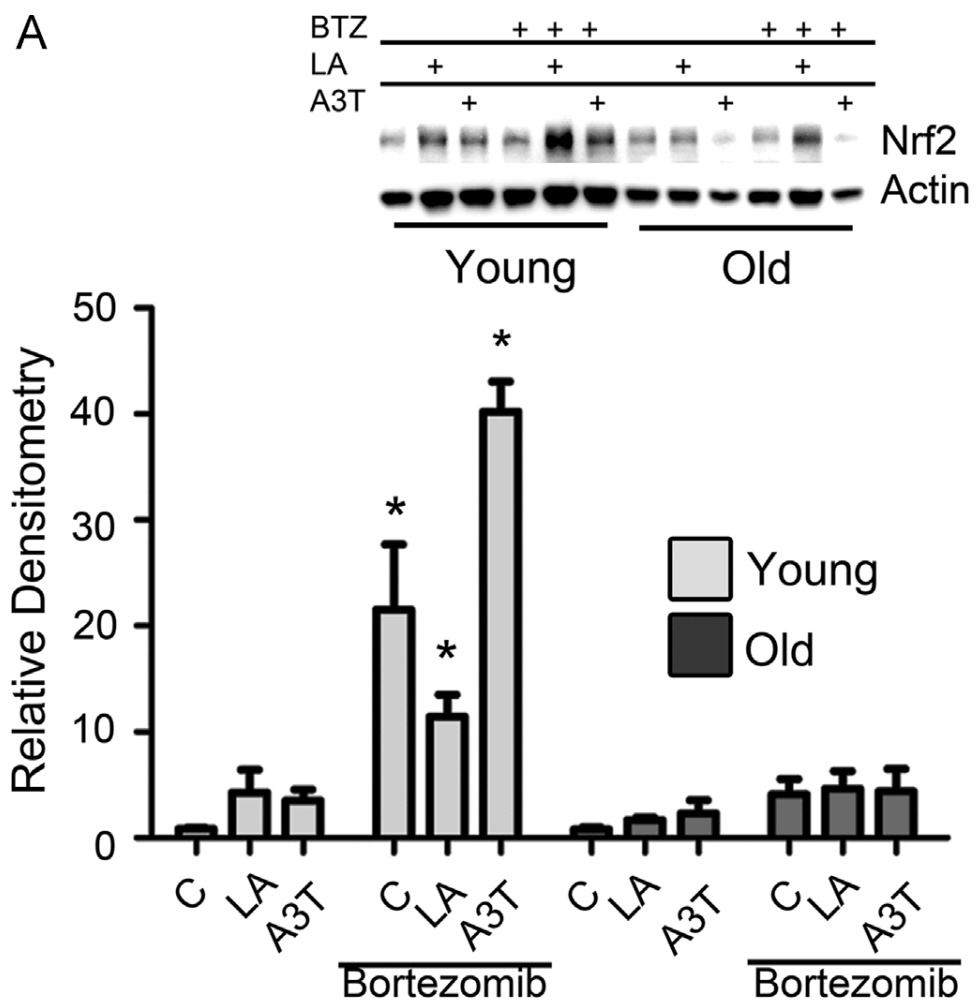


Figure 2.3. Stress response of the ARE is attenuated with age. Hepatocytes isolated from young and old rats were transfected with the ARE-Firefly luciferase and Renilla-PLG4 (control) luciferase reporter vectors. After 24h, cells were treated with vehicle control (C), 100 mM lipoic acid (LA), or 50 mM A3T for 16h, then lysed and assayed for luciferase activities (N = 3; *p < 0.05). Control values were normalized to 1.0 for both young and old in order to show the relative increase.



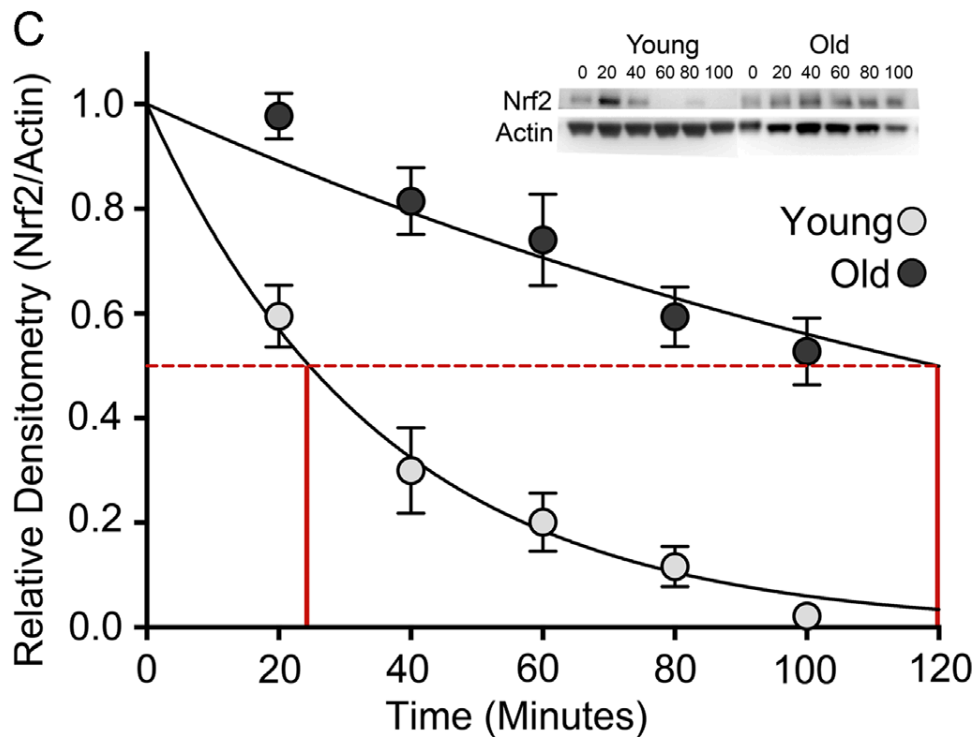


Figure 2.4. Both Nrf2 synthesis and degradation decline with age. (A) Hepatocytes from young and old rats were treated with 50 mM A3T, 100 mM lipoic acid (LA), and/or 100 nM bortezomib for 6h. Nuclear fractions were isolated from the harvested samples and assayed for Nrf2 content. Samples were quantitated by densitometry relative to the loading control Actin. Statistical analysis was done by two-way ANOVA and Tukey's post-hoc method. * $p < 0.05$ vs young control without BTZ (N = 3). (B) Graphic representation of Keap1 immunoblots using liver tissue from young and old rats N = 4; * $p < 0.05$. Actin loading control is from the same western blot membrane shown in Fig. 1A. (C) Half-life of Nrf2 protein was determined by transfecting primary hepatocytes from young and old rats with the pHA-Nrf2 expression vector, then adding 100 mM cycloheximide 20h post-transfection. Samples were taken every 20 min and protein was extracted. Nrf2 protein levels were determined by immunoblot, quantified by Image J, and used to create the graph (N = 3).

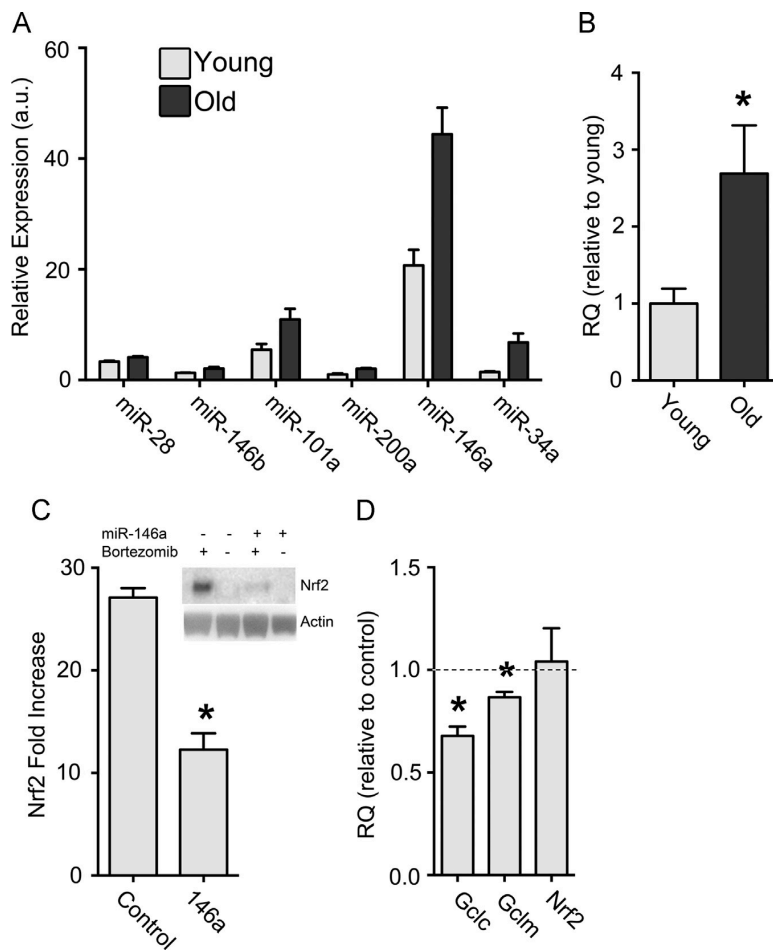


Figure 2.5. Increased miR-146a with age reduces Nrf2 protein levels. (A) Liver tissue from 6 young (6 months) and 6 old (24 months) rats were analyzed on a miRNA array. Results were limited to p values of less than 0.05 and corrected for multiple comparisons. The miRNAs predicted and/or experimentally determined to modulate Nrf2 are shown. (B) Liver tissue from 6 young and 6 old rats were analyzed by RT-qPCR for levels of miR-146a. The difference between young and old is significant by Student's t-test $*p < 0.05$. (C) Hepatocytes isolated from young rats were transfected with rno-miR-146 a miRNA mimic or scrambled RNA oligomer, treated with BTZ for 6h, and compared to vehicle-treated hepatocytes (N = 3 for each condition). Nrf2 protein levels and loading control Actin were measured by immunoblot analysis and quantitated by densitometry. Graph shows Nrf2 fold increase over non-BTZ-treated cells (= 1) ($*p < 0.05$). (D) mRNA levels were measured and compared to hepatocytes transfected with a scrambled RNA oligomer (= 1.0). Although no significant difference was seen in the RQ of the Nrf2 mRNA, both Gclc and Gclm decrease significantly, $*p < 0.05$.

Chapter 3

Glutathione Maintenance Mitigates Age Related Susceptibility to Redox Cycling Agents

Nicholas O. Thomas, Kate P. Shay, Amanda R. Kelley, Judy A. Butler, and Tory M. Hagen

Redox Biology

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3.1 Abstract

Isolated hepatocytes from young (4-6 mo) and old (24-26 mo) F344 rats were exposed to increasing concentrations of menadione, a vitamin K derivative and redox cycling agent, to determine whether the age-related decline in Nrf2-mediated detoxification defenses resulted in heightened susceptibility to xenobiotic insult. An LC₅₀ for each age group was established, which showed that aging resulted in a nearly 2-fold increase in susceptibility to menadione (LC₅₀ for young: 405 μ M; LC₅₀ for old: 275 μ M). Examination of the known Nrf2-regulated pathways associated with menadione detoxification revealed, surprisingly, that NAD(P)H:quinone oxido-reductase 1 (NQO1) protein levels and activity were induced 9-fold and 4-fold with age, respectively ($p=0.0019$ and $p=0.018$; $N=3$), but glutathione peroxidase 4 (GPX4) declined by 70% ($p=0.0043$; $N=3$). These results indicate toxicity may stem from vulnerability to lipid peroxidation instead of inadequate reduction of menadione semi-quinone. Lipid peroxidation was 2-fold higher, and GSH declined by a 3-fold greater margin in old versus young rat cells given 300 μ M menadione ($p<0.05$ and $p\leq 0.01$ respectively; $N=3$). We therefore provided 400 μ M N-acetyl-cysteine (NAC) to hepatocytes from old rats before menadione exposure to alleviate limits in cysteine substrate availability for GSH synthesis during challenge. NAC pretreatment resulted in a >2-fold reduction in cell death, suggesting that the age-related increase in menadione susceptibility likely stems from attenuated GSH-dependent defenses.

This data identifies cellular targets for intervention in order to limit age-related toxicological insults to menadione and potentially other redox cycling compounds.

Keywords: Glutathione; Menadione; Redox-cycling; Detoxification Capacity; Aging

Chemical Compounds: BSO (PubChem CID: 119565), DMF (PubChem CID: 6228), NAC (PubChem CID: 12035), Menadione (PubChem CID: 4055)

Abbreviations: ARE (Antioxidant Response Element), BSO (Buthionine-S,R-Sulfoximine), CPR (Cytochrome P450 reductase), DCPIP (Dichlorophenolindophenol), GPX4 (Glutathione Peroxidase 4), LDHA (Lactate dehydrogenase A), LOO[·] (Lipid peroxide), MDA (Malonyldialdehyde), NQO1 (NAD(P)H:quinone oxido-reductase 1), NQO2 (NAD(P)H:quinone oxido-reductase 2), Nrf2 (Nuclear factor (erythroid-derived 2)-like 2), RIPA (Radioimmunoprecipitation assay), TRE (TPA-Response Element)

3.2 Introduction

A major hallmark of aging, and a key driver for the onset of age-related pathophysiologies across multiple species, including primates, is the disruption of cellular redox homeostatic mechanisms that protect against a variety of environmental, oxidative, pathological, and toxicological insults [3,8,9,12,175,176]. Nrf2-dependent phase II detoxification mechanisms in particular, tend to decline with age [56,177–181]. The age-related decrease in these detoxification pathways and ensuing increase of reactive oxygen and nitrogen species (ROS and RNS) is well established and is causally linked to various pathologies such as cardiovascular and neurodegenerative diseases, cancer and diabetes [6,10,13,15,130,182–186]. The mechanisms associated with this loss are poorly understood; however, we have found that hepatic Nrf2 protein synthesis declines with aging and that phase II detoxification gene expression is limited [56,58,59]. However, despite the age-related decline in basal expression of Nrf2 regulated detoxification enzymes, it remains unknown whether this loss magnifies the toxicological exposure effect of ROS and RNS and xenobiotics detoxified through these pathways. Of particular interest to this work are the age-associated changes to resilience against acute exposure to a redox cycling challenge.

Redox cycling compounds are prooxidant catalysts, which facilitate the transfer of electrons onto oxygen to produce reactive oxygen species (ROS) [187,188]. These compounds are highly abundant as substituents in xenobiotic compounds (e.g. redox active metals and pesticides) [189–193], redox active pharmacophores (e.g. anesthetics) [194], and especially, their use in pharmacotherapeutic drugs (e.g. menadione, anthracycline, adriamycin, and doxorubicin) [195–199]. A decreased capacity to detoxify redox cycling agents in the liver could potentially increase vulnerability to xenobiotic exposures, as well as limiting some medical treatment options such as antibiotics and anti-cancer chemotherapeutics [129,200–202]. This is of particular importance as cancer incidence increases exponentially with age [203]. Thus, it is important to determine whether there is an age-related decline in detoxification of redox cycling compounds and if so, which of these types of drugs, toxins, or environmental xenobiotics have a heightened toxicity profile.

In order to test our hypothesis that there is an age-related decline in resilience to redox cycling compounds in the liver, we employed an acute menadione challenge. Menadione, a derivative of vitamin K and a redox cycling agent, and its mechanism of action is well characterized. Herein, we show that hepatocytes from aged rats are acutely more susceptible to menadione insult. Moreover, while certain detoxification enzymes involved in menadione metabolism actually increase with age, the observed age-associated vulnerability to menadione

appears to stem from a marked attenuation of Nrf2-regulated GSH-dependent detoxification pathways.

3.3 Materials and methods

3.3.1 Reagents

NAC (Cat# 616-91-1), menadione (Cat# 58-27-5), NADPH (Cat# 2646-71-1), dichlorophenolindophenol (DCPIP; Cat# 620-45-1), and protease inhibitor cocktail (Cat# P8340) were ordered from Sigma-Aldrich (St. Louis, MO). Collagenase type IV was purchased from Worthington Biochemical Corporation (Lakewood, NJ). PVDF transfer membrane was purchased from Millipore (Billerica, MA). Dicumarol (Cat# 66-76-2) was ordered from Calbiochem (Darmstadt, Germany).

3.3.2 Animals

Both young and old male F344 rats were from the National Institute on Aging animal colonies. The rats were housed in the Linus Pauling Institute animal facility and allowed to acclimatize for at least 1 week prior to any experimentation. Animals were maintained on a 12 h light cycle (7am to 7pm) and fed standard chow *ad libitum*. All animal work was approved and in

accordance to IACUC guidelines (Assurance Number: A3229-01). The AAALAC-accredited Laboratory Animal Resources Center (LARC) provided management and veterinary care.

3.3.3 Hepatocyte Isolation

Hepatocyte isolation was performed as described previously [120]. Briefly, after animal sacrifice via IACUC-approved protocols, the liver was perfused via a cannula in the portal vein with Hank's balanced salt solution, pH 7.4. Following removal of blood, liver cells were disassociated using collagenase solution (1 mg/ml). The resultant cell suspension was filtered through sterile gauze to remove connective tissue and debris. Parenchymal cells were isolated using gravity filtration and washed three times with Krebs-Henseleit solution, pH 7.4. Cells were resuspended in Krebs-Henseleit solution and placed in a round bottom flask and rotated at room temperature for 1 h before cell count and viability were assessed using trypan blue exclusion.

3.3.4 Cell and Tissue Lysates

For whole cell lysates, suspended cells were harvested by centrifugation at 100 x g, washed in Krebs-Henseleit solution, pH 7.4, and sonicated in lysis buffer (50 mM Tris, pH 7.5, containing 1% NP-40 (v/v), 100 mM NaCl, 2 mM EDTA,

2 mM sodium ortho-vanadate) with added protease inhibitors. For tissue, lysates were obtained as previously described by Siegel et al. [204]. Briefly, tissue was homogenized using a dounce homogenizer in RIPA buffer with a volume to weight ratio of 5:1. The homogenate was sonicated 3 times and centrifuged for 15 minutes at 10,000 x g centrifugation (4°C) before supernatants were collected for assays. For the NQO1 assay, supernatants from tissue lysates were subjected to an additional ultracentrifugation step (30,000 x g for 1h at 4°C). Protein concentrations of samples were determined either by the Bradford Assay (Cat# 500-0006, BioRad) or Pierce 660 nm assay (Cat# 22660, Thermo Scientific).

3.3.5 Assessment of Menadione Toxicity

Hepatocytes were diluted to 4×10^6 cells/mL using Krebs-Henseleit solution, pH 7.4, and rotated on a MACSMIX (Miltenyi Biotec) rotator placed in a cell culture incubator (5% CO₂ at 37°C) to maintain the cells in suspension. Hepatocytes were treated with increasing concentrations of menadione (0, 100, 200, 300, 400, 500, and 600 μM) for 2 h before being assayed for viability using trypan blue exclusion. Menadione was solubilized in dimethylformamide (DMF). DMF was also used as the vehicle control and total DMF in treated cell suspensions was 0.05% by volume for all treatment experiments.

3.3.6 NQO1 Activity Assay

NQO1 activity of samples was assayed as described previously by Siegel et al [204]. Briefly, tissues from young and old animals were prepared as described above before being assayed for the NAD(P)H-dependent reduction of DCPIP by NQO1 in the presence and absence of dicumarol (a reversible NQO1-specific inhibitor). DCPIP reduction was assayed using a DU800 spectrophotometer at 600 nm over 1 minute, and NQO1 activity was measured as the dicumarol inhibitable portion of the reduction. Final concentrations of reagents in reaction solution were 0.2 mM NAD(P)H and 40 μ M DCPIP with and without 20 μ M dicumarol.

3.3.7 Malondialdehyde Quantification

Measurement of the lipid peroxidation product, malonyldialdehyde (MDA), was performed as previously described by Wong et al. [205] and modified by Sommerburg et al. [206]. Briefly, 200 μ L of suspended cells (4×10^6 cells/mL) was mixed with 750 μ L of 440 mM phosphoric acid, 250 μ L of 42 mM thiobarbituric acid (TBA), and 300 μ L of water prior to being boiled for 1 h. The reaction was quenched by placing samples into an ice bath. Samples then had an equal volume (1.5 mL) of 1 M NaOH added before being centrifuged at 16,000 x g for 5 minutes at 10° C. Malondialdehyde was separated from other metabolites by HPLC using

a Luna C18(2)] Phenomenex #00G-4252- E0) column in isocratic mode (25 mM potassium phosphate buffer, pH 6.5/methanol [50:50] as eluents) and detected by fluorescence (excitation, 532 nm; emission, 553 nm). Malondialdehyde was quantified relative to a tetramethoxypropane (TMP) standard curve.

3.3.8 Immunoblotting

Lysates were prepared as described above, sonicated, and proteins were solubilized for SDS-PAGE in Laemmli loading buffer containing SDS. Samples were heat-denatured for 5 min at 100°C. Normalized amounts of protein (30 µg/lane) were run on SDS-PAGE and transferred to PVDF membranes with a semi-dry blotter. Membranes were blocked in PBS containing 1% Tween-20 with either 5% nonfat dry milk or 3% BSA, incubated with primary antibodies for 2 h at room temperature, washed, and incubated with secondary antibodies for 1 h at room temperature, washed, incubated with chemiluminescence reagents, exposed to film, and developed. Images shown were cropped for size/space considerations. Antibodies made to the following proteins were used: GPX4 (Protein Tech - Cat# 14432-1-AP), NQO1 (Abcam - Cat# ab2346), Cytochrome P450 Reductase (CPR, EC 1.6.2.4; Abcam - Cat# 13513), Lactate Dehydrogenase A (LDHA; Cell Signaling Technology - Cat# 2012) and Actin (Sigma-Aldrich - Cat# A5044). Blots were densitometrically analyzed with ImageJ software from NIH.

3.3.9 Glutathione Analysis

Glutathione (GSH) content of suspended cells was determined according to the methods of Fariss and Reed [207] as modified by Smith et al. [56]. Briefly, suspensions were homogenized in an equal volume of 10% (w/v) perchloric acid (PCA) containing 10 mM EDTA. After deproteinization, 200 μ L of the supernatant containing internal standard (γ -glutamyl glutamate) was mixed with 50 μ L iodoacetic acid (100 mM dissolved in 0.2 mM *m*-cresol purple) and the pH was adjusted to 10 by using KOH–KHCO₃ buffer (2 M KOH:2.4 M KHCO₃). Samples were placed in the dark, at room temperature for 1 h. The resulting *S*-carboxymethyl derivatives were subjected to further chemical modification by addition of an equal volume of 1-fluoro-2,4-dinitrobenzene (1% v/v in absolute ethanol). The solution was incubated overnight in the dark and at room temperature. Samples (75 μ L) were separated by HPLC using a Thermo Scientific (Eugene, OR) APS-2 Hypersil column and detected on a Shimadzu (Kyoto, Japan) SPD-10AVP UV-VIS spectrophotometer with the absorbance set at 365 nm. Quantitation was obtained by integration relative to GSH and GSSG standards.

3.3.10 Statistical Analysis

All statistical analysis was performed using Excel (Microsoft, Inc.) and Prism 6 and 7 (GraphPad Software, Inc.). Error bars represent standard error of the mean. For comparisons between two samples, two-sided Student's t-test was used. Differences between samples that resulted in a p-value of ≤ 0.05 were considered statistically significant. Statistical analyses for multiple comparisons were evaluated by Student's t-test with the Sidak-Bonferroni post-hoc method.

3.4 Results

3.4.1 Acute menadione challenge causes hepatocellular damage and loss of viability.

To establish the relative age-associated susceptibility to menadione challenge, aliquots of freshly isolated hepatocytes from young and old rats were incubated with increasing concentrations of menadione, and viability was assessed after 2 h of challenge. Results in Figure 1 show that regardless of age, increasing concentrations of menadione corresponded with heightened indices of cell damage and loss of viability. For hepatocytes from young rats, the lowest concentration of menadione employed (100 μM) resulted in no changes in toxicity versus vehicle control; in contrast, hepatocytes isolated from old rats lacked any resilience to menadione, demonstrating viability loss at all concentrations tested. The LC_{50} for menadione was calculated to be 405 μM and

275 μM for young and old rat hepatocytes, respectively (Figure 1). These results indicate a significantly higher susceptibility to menadione with age ($p < 0.05$, $N = 3$), and suggest that basal cellular defenses to this redox cycling agent are compromised.

3.4.2 Increased vulnerability to menadione with age is not caused by a loss in NQO1 or increase in Cytochrome P450 Reductase (CPR).

Considering the mechanisms associated with its detoxification (Figure 2), heightened age-dependent vulnerability to menadione may stem from altered activities of one or more antioxidant or phase II detoxification enzymes. As almost all of these enzymes are primarily regulated by Nrf2 [45,47,112,208–211], we sought to define potential lesions in menadione detoxification, which might serve as the basis for its enhanced toxicity with age.

CPR converts menadione from the quinone to semiquinone state; therefore, any age-related increase in the reductase would lead to higher levels of redox cycling. However, we found that the CPR protein decreased by ~ 2 -fold in hepatocytes from old animals (Figure 3A, $p = 0.0137$, $N = 3$). These results suggest that, if anything, there may be less menadione redox cycling with age progression.

Because CPR could not account for the elevated toxicity from menadione, we examined the hepatic levels and activity of NQO1, as this enzyme catalyzes the two-electron reduction of menadione to the fully reduced hydroquinone state (Figure 2). Full reduction limits redox cycling and initiates menadione detoxification and removal from cells. Rather than the hypothesized decline in NQO1 levels with age, we observed that NQO1 protein content and enzyme activity in livers from old rats increased markedly compared to that seen in young animals. As shown in Figure 3B, NQO1 activity was nearly 4-fold higher with age ($p = 0.0185$, $N=3$), which is consistent with heightened hepatic protein content of the enzyme (9.3-fold increase, $p = 0.0019$, $N=3$) in Figure 3C. While this significant elevation in steady-state NQO1 activity may represent an adaptation toward the heightened pro-oxidant cellular milieu of the aging rat liver, these results nevertheless suggest that NQO1 is not likely a part of the mechanism associated with vulnerability to menadione.

3.4.3 Age-related decline in hepatic GSH and GPX4 contribute to menadione-mediated cytotoxicity.

Menadione and the reactive oxygen species initiated by its redox cycling are primarily detoxified by GSH-dependent mechanisms (cf. Figure 2), which we previously reported to decline significantly with age [56]. In order to determine whether hepatic GSH levels become limiting under menadione insult,

hepatocytes from young and old animals were treated with menadione at approximately the LC_{50} for old rats (300 μ M). Results show that menadione rapidly eliminates GSH in both age groups, but it caused a markedly greater loss of GSH hepatocytes from old rats. As shown in Figure 4, menadione resulted in >80% decline in GSH within one minute in old rat hepatocytes. The menadione-mediated loss of GSH was significantly less severe in cells from young animals (~60% decline). GSH levels at all time points were significantly different between young and old ($p \leq 0.01$, $N=3$). Moreover, GSH levels in the young rat hepatocytes never decreased below a threshold of ~35%. Thus, reserves of hepatic GSH are more rapidly and extensively lost with age, and this precipitous decline in GSH precedes indices of cellular damage and loss of viability.

To further determine age-associated differences in GSH-dependent detoxification of menadione, we examined hepatic concentrations of glutathione peroxidase 4 (GPX4). This enzyme is a primary protectant against phospholipid hydroperoxides, and is also known to play a significant role in protection against redox cycling [116,117]. Western blot analysis revealed that GPX4 protein levels were significantly lower in old than in young hepatocytes (Figure 5A; 70%, $p = 0.0043$, $N=3$), which is consistent with reports of age-related loss in GPX4 levels in multiple tissues, including the liver [115,212].

Because GPX4 chiefly protects against lipid peroxidation, we measured hepatic levels of malondialdehyde (MDA), a biomarker of the oxidation of polyunsaturated lipids, through its derivatization with thiobarbituric acid (TBA). Because TBA derivatization may overestimate lipid peroxidation levels, we employed a well-characterized HPLC method to minimize ex vivo artifacts so that relative differences in lipid peroxidation with age and menadione could be discerned. [213] It is interesting to note that, without menadione, no age-associated differences in MDA levels were observed, indicating that basal lipid oxidation and its detoxification are maintained despite the age-related loss of GPX4 (Figure 5B). However, exposure to menadione at the approximate LC₅₀ for old rat hepatocytes (300 μ M), resulted in a rapid appearance of MDA in cells from both age groups (Figure 5C). Hepatocytes from aged rats were demonstrably more vulnerable to menadione-mediated lipid peroxidation. Initial increases in MDA levels for cells from both young and old animals was observed, followed by a brief stabilization of MDA levels between the 2 and 5 minute time points. After this, MDA levels in cells from young animals returned to baseline. In contrast, hepatocytes from old rats displayed a rapid increase in MDA levels after the 2 through 5-minute stabilization period. After 15 minutes, MDA levels were 147.4 ± 13.8 and 274.2 ± 35.1 pmol/mg protein in liver cells from young and old animals, respectively. Moreover, lipid peroxidation levels in hepatocytes from young animals never increased by more than 5%, whereas the extent of lipid oxidation in hepatocytes isolated from old rats were 78% higher

and still rising 15 minutes after menadione challenge. The divergence between young and old is striking and aligns with loss of GPX4, as well as the appearance of cellular damage and loss of viability.

3.4.4 Age-related menadione toxicity is reversed by GSH precursors.

Because GPX4 works at the expense of GSH, and GSH is also lowered with age in our model, we hypothesized that providing cells with NAC should relieve the cysteine substrate limitations for GSH synthesis, and thus increase resistance to menadione insult. A 1 h pretreatment with NAC (400 μM) before menadione exposure significantly increased resistance to menadione in old rat hepatocytes. In fact, our data show that NAC treatment reversed the increased age-associated vulnerability to menadione (Figure 6, $p=0.003$, $n = 5$), making this indistinguishable from 300 μM menadione-treated hepatocytes from young animals (Figure 1). Co-pretreating with buthionine sulfoximine (BSO; 400 μM for 1 h), a specific GSH synthesis inhibitor, eliminated the ability of NAC to rescue hepatocyte viability, which serves as a proof of concept that GSH is the key component.

3.5 Discussion

The aging process leads to disparate changes in hepatic detoxification capacity. For example, many phase I enzymes are unaffected by age, while phase II detoxification processes are more variable [214–216]. Our data exemplifies this with NQO1 levels and activity increasing significantly in aging hepatocytes, but GPX4 appreciably declines. Therefore, while a general age-related loss in basal detoxification capacity is apparent, it is not clear if this decline is at least partly compensatory, or leads to vulnerability to certain classes of xenobiotic compounds. Our present study is important as our data supports the concept that, at least for redox cycling compounds, (e.g. menadione) there is a profound susceptibility with age.

The current study focused on susceptibility to acute menadione insult using freshly isolated hepatocytes from young and old rats. This cell model is appropriate for understanding aging differences in detoxification because these cells retain their aging phenotype with respect to stress resistance. It also allows for a direct assessment of potential age-induced differences in hepatic detoxification capacity at the time of animal sacrifice [120]. Using this hepatocyte model, we previously showed that steady-state Nrf2 levels significantly decline both in hepatocytes and in the aging rat liver [56,58,59].

We used menadione in the current study because most of the enzymes involved in its detoxification are Nrf2-dependent. For example, NQO1 maintains

menadione in its fully reduced hydroquinone state [187,188,217] prior to its further metabolism and removal from the cell (*cf.* Figure 2). Additionally, because menadione is a potent redox cycling agent, its mode of acute toxicity results from a cascade of redox cycling, lipid peroxidation, and membrane damage [188,218–221]. As such, we chose to examine GSH and GPX4, the principal glutathione peroxidase that is localized to membranes, as the primary players in preventing cell damage from acute menadione exposure [222–225]. While GPX1 also plays a major role in peroxide detoxification, it is localized to the cytosol and thus is not the initial detoxifier of menadione-induced lipid peroxidation. Thus, xenobiotic disposition and the extent of acute oxidative damage ensuing from menadione exposure are largely dependent on the hepatocellular activity of NQO1, GSH, and GPX4—all of which are Nrf2-regulated mechanisms.

Despite this common tie to Nrf2 associated gene transcription, we found that aging does not uniformly affect all of the enzymatic components involved in menadione detoxification. For example, we observed that hepatic levels of NQO1 and its activity significantly increase with age, while CPR activity is diminished. Overall, these results could be interpreted as an attempt by the hepatocyte to limit levels of the semiquinone species, thereby lowering the rate of redox cycling. However, this protective adaptation against menadione insult in the aging rat liver is counterbalanced by the significant loss of GSH levels and GSH-

dependent detoxification enzymes (e.g. GPX4). The reason(s) why certain Nrf2-dependent genes are affected with age while other genes do not change or decline are not presently known. With respect to NQO1, it has been reported that with age, NQO1 levels and/or activity decline [226], increase [227], or remain similar to that seen in young [228]. These results may stem from the varied methods employed to monitor NQO1. We used the reduction of DCPIP as first developed by Ernster et al. [229] and recently optimized by Ross et al. [204]. This method has the benefit of highly purifying the cytosolic fraction through ultracentrifugation and doesn't assay the reduction of menadione-cytochrome c, which has confounding NQO2 enzymatic activity. Additionally, the NQO1 promoter region contains an embedded TRE (TPA-response element) within the ARE enhancer [230,231] and demonstrates higher recruitment and binding efficiency for Nrf2 both for basal and induced transcription [54,121]. These factors may account for the increase we observed in spite of Nrf2 decline. Regardless of the precise mechanism, it is clear that NQO1 is not involved in the age-related enhanced vulnerability to menadione that we observed in this study.

It is equally clear that GSH-dependent defenses are compromised in hepatocytes from aging rats. We and others have previously shown that hepatic GSH declines with age [56,131,134,232–234]; however, GSH remains at millimolar levels even in very old animals. Thus, it was not known prior to the present study whether the liver is more susceptible to menadione insult that would result from

attenuated GSH concentrations. Our results show that menadione causes a rapid loss of GSH in hepatocytes from both young and old rats, but the rate and degree of GSH loss is more extensive in older animals. This lack of capacity to maintain cellular GSH appears to be the most important factor leading to lost resiliency against menadione challenge with age. This concept is buttressed by our results using NAC and NAC+BSO to modulate GSH prior to menadione insult. In particular, NAC, which supplies L-cysteine for GSH synthesis [235–237], essentially abrogates the increased vulnerability to menadione in aged rat hepatocytes. Taken together, the inability to supply GSH for detoxification, along with diminished GPX4, potentially sets the stage for the aging cell to be susceptible to a variety of toxins, oxidants, and environmental mutagens.

An additional aspect revealed in this study is that GSH levels in young rat hepatocytes never decreased below ~35% of initial levels versus the 90% lost by old. This suggests that when the menadione-mediated oxidation crosses a critical threshold of GSH, cellular toxicity rapidly ensues. Because mitochondria contain a separate GSH pool that approximates this threshold level, it is enticing to suggest that maintenance of mitochondrial GSH is particularly important to resist menadione insult. In this regard, loss of mitochondrial GSH has been demonstrated to cause a decline in mitochondrial membrane potential [70,77,220,238,239], inducing calcium overload and initiating necro-apoptotic

pathways leading to cell death [240–244]. We are currently investigating the role of mitochondrial GSH in sensitizing the cell toward oxidative insult in aging.

Finally, the results presented in this study identify a specific cellular target to potentially improve detoxification and xenobiotic metabolism. There is a significant clinical history of using NAC to limit toxicity from acute exposure to drugs and toxins [245–247]. Our data suggest that a similar preventative strategy of NAC administration may be warranted to increase resistance to xenobiotic and drug toxicity in older adults. GSH is a principal detoxicant for environmental xenobiotics, pharmaceuticals, air pollutants, and heavy metals. Providing NAC to increase substrate supply of cysteine may circumvent the age-related decline in GSH synthetic enzymes that attenuation of Nrf2 engenders. Thus, using NAC as a prophylactic instead of an intervention may allow GSH levels to be maintained for detoxification in older adults.

Acknowledgments

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3.6 Figures

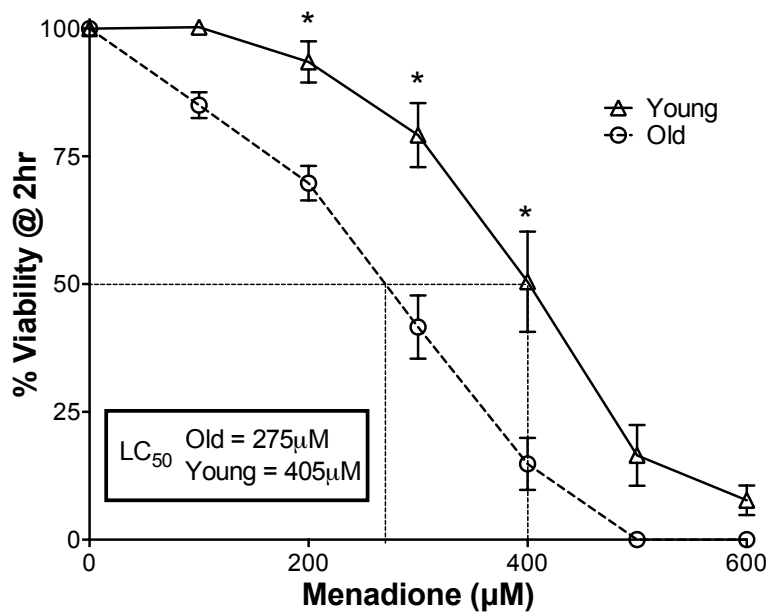


Figure 3.1. Acute menadione toxicity is enhanced with age. Isolated hepatocytes from young and old rats were treated with increasing amounts of menadione (0 - 600 µM). Viability was determined by trypan blue exclusion 2 h after exposure. N=3, *p<0.05.

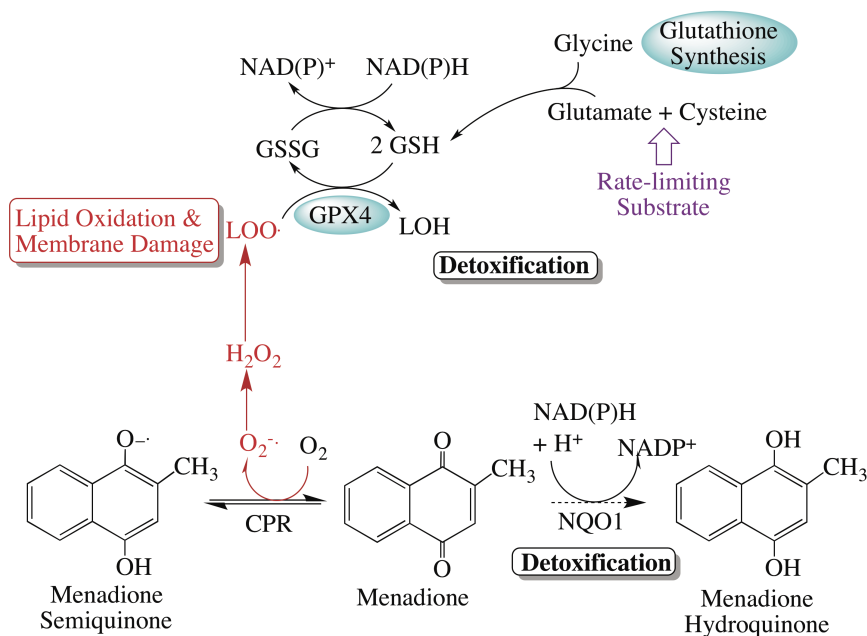


Figure 3.2. Menadione detoxification scheme. Menadione is a potent redox cycling agent which cycles between the quinone and semi-quinone states, producing superoxide, which may eventually lead to oxidative and nitrosative damage. NQO1 detoxifies the menadione quinone by reducing it to the hydroquinone, which is then eliminated. The major resultant oxidative damage from redox cycling is lipid peroxidation (LOO \cdot), which is primarily detoxified by the glutathione-dependent GPX4 enzyme.

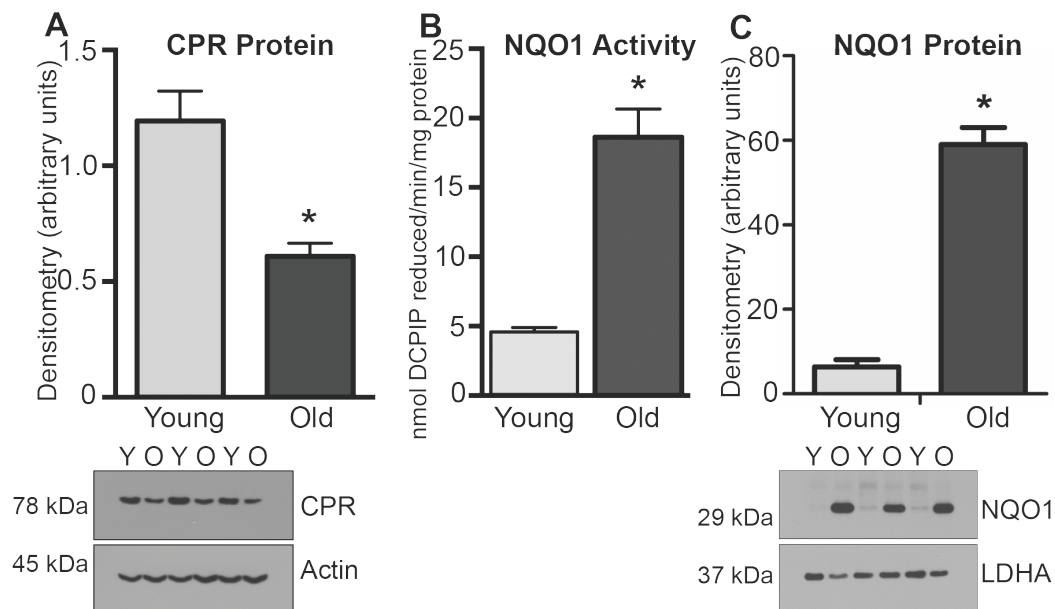


Figure 3.3. CPR protein levels decrease while NQO1 protein levels and activity increase with age. CPR protein levels (A), and NQO1 activity (B) and protein levels (C) in liver tissue homogenates from young (Y) and old (O) animals were evaluated. N=3 for A, B and C, *p = 0.0137, 0.0185 and 0.0019 respectively.

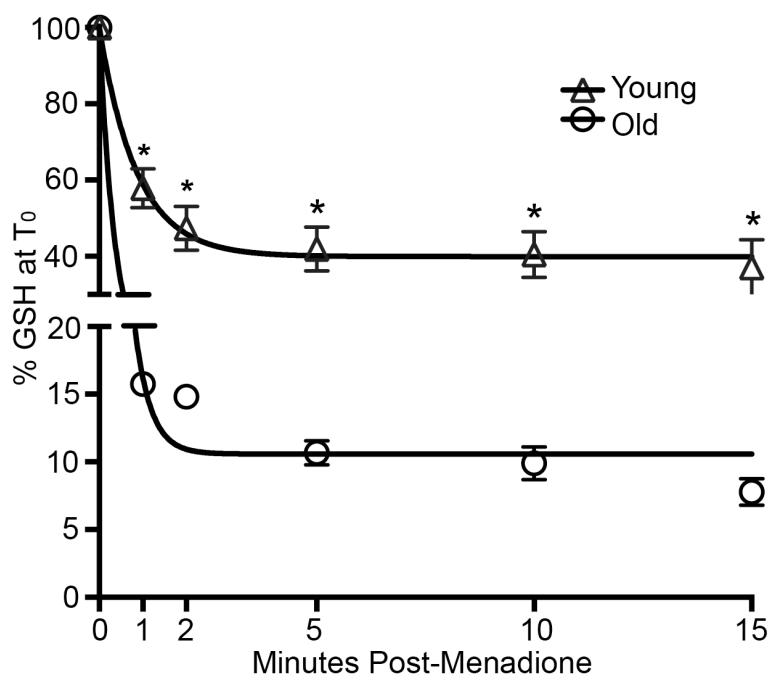


Figure 3.4. GSH loss during menadione treatment is accelerated with age. Hepatocytes from young and old rats were treated with 300 μM menadione over a 15 minute time course and assayed for GSH content. N=3, *p<0.01.

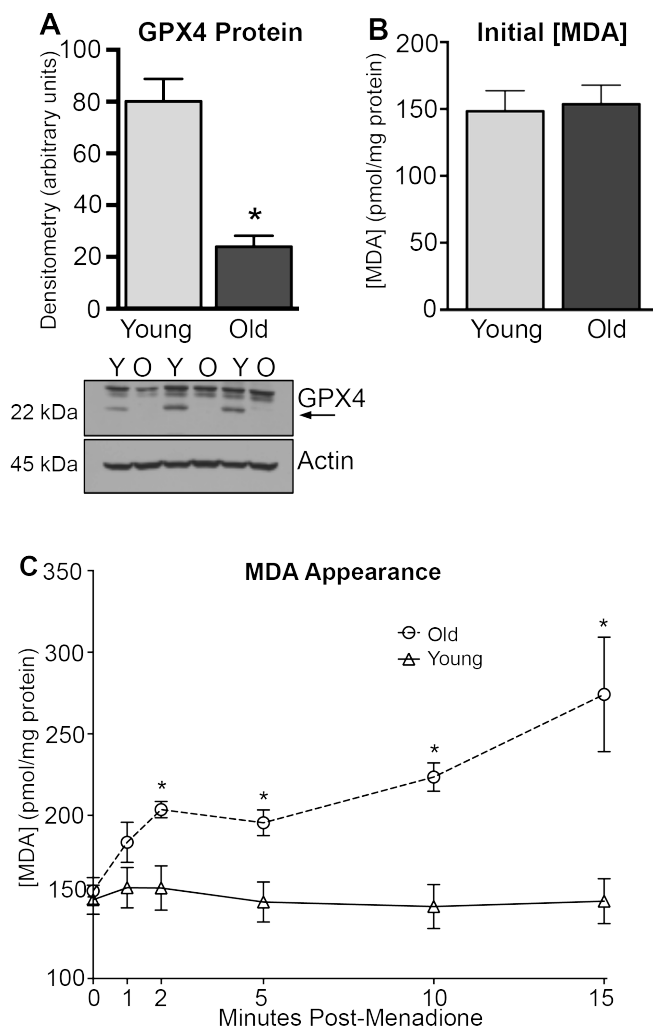


Figure 3.5. GPX4 protein levels are significantly reduced with age which correlates with an increased appearance of menadione-induced MDA. Hepatocytes from young (Y) and old (O) animals were evaluated for GPX4 protein levels by western blot (A). Basal MDA content (B) and MDA accumulation (C) were assayed after menadione treatment. N=3 for A, B and C, *p=0.0043, >0.05, and <0.05 for A, B, and C respectively.

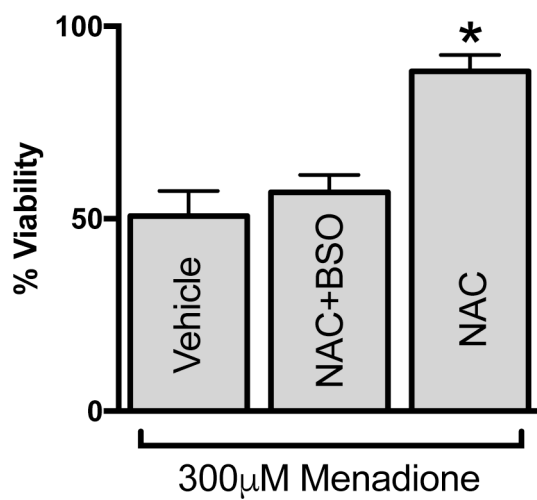


Figure 3.6. Pretreating cells with NAC prior to menadione exposure improves resistance. Hepatocytes from old animals were pretreated with vehicle (water), 400 μM NAC, or 400 μM NAC & 400 μM BSO for 1 h prior to addition of 300 μM menadione. Viability was determined 2 h later. N=5, *p=0.003 versus vehicle.

Chapter 4

The Age-Related Loss of Mitochondrial Glutathione Exacerbates Menadione-Induced Inhibition of Complex I

Nicholas O. Thomas, Kate P. Shay, Amanda R. Kelley, Judy A. Butler, and Tory M. Hagen

4.1 Abstract

In order to determine the role of mitochondrial GSH (mGSH) loss in increased susceptibility to xenobiotic insult with age, markers of mitochondrial function were measured in intact and digitonin permeabilized isolated hepatocytes from young (4-6 mo) and old (24-26 mo) F344 rats under a redox cycling (300 μ M menadione) challenge (the \sim LC₅₀ for old). While the rate of mGSH loss under menadione challenge was similar in both age groups, the difference in basal mGSH with age (68 vs 36 nmol GSH/mg protein, N=1) ultimately resulted in a 50% loss of mGSH in old rat hepatocytes versus only 28% in young within 10 minutes of exposure. Examination of mitochondrial membrane potential ($\Delta\psi_m$), which is acutely regulated by mGSH content, showed a distinct loss in basal $\Delta\psi_m$ (\sim 21%). Additionally, within five minutes of menadione treatment, $\Delta\psi_m$ was not markedly reduced in young but had collapsed and passed the threshold for mitochondrial permeability transition pore opening (\sim 100 mV) in old rat hepatocytes. Further characterization demonstrated that basal respiration and respiratory reserve capacity, indicators of cellular bioenergetic capacity, were both significantly reduced upon menadione treatment in old rat hepatocytes (34% and 72% respectively, N=4, $p < 0.05$) but not in young. These results suggested that the age-related difference in mitochondrial function under menadione challenge might stem from mGSH regulated ETC components. We therefore examined proton leak, complex I, and complex II contributions to

mitochondrial oxygen consumption rates under menadione challenge in both age groups. Results showed no marked effect in young rat hepatocytes, while old rat hepatocytes demonstrated significant increases in proton leak and complex II contributions to oxygen consumption rate (4.2 and 3.6-fold respectively, N=4, $p<0.05$), along with a significant decline in complex I contribution (4.8-fold, N=4, $p<0.05$). This data clearly demonstrates an age-related increase in mitochondrial susceptibility to menadione challenge, particularly in complex I, and provide a plausible mechanism that links this vulnerability to age-related mGSH perturbations.

4.2 Introduction

Aging is characterized by a decline in cellular redox homeostasis and detoxification capacity, which is coincident with an increase in risk for age-related pathophysiologies [8,9,12,56,106,130,178,181,248]. An underlying factor that has been implicated in aging and age-related diseases is mitochondrial dysfunction and decay [249]. Characteristics of this decay include a decreased membrane potential ($\Delta\psi_m$), basal respiration rate, and respiratory reserve capacity, as well as increases in oxidant production, mitochondrial DNA damage, and susceptibility to the formation of the mitochondrial permeability transition pore (MPTP) leading to apoptotic initiation [70,78,250]. While many possible factors could be involved in driving mitochondrial dysfunction with age, this work will focus on the ramification of the age-related loss of mitochondrial glutathione and glutathione dependent detoxification pathways. Glutathione (GSH) is a critical regulator of mitochondrial ATP production in that it regulates critical cysteine sulfhydryl oxidation statuses, which in turn influence both the NADH and FADH₂ generation and electron flow through the ETC [72,92]. Finally, GSH constitutes one of the primary defenses against oxidative damage both as an antioxidant and as a substrate for multiple detoxification enzymes such as the glutathione-S-transferases (GSTs), glutaredoxins (Grxs), and glutathione peroxidases (GPxs). Thus, mitochondrial GSH (mGSH) is critical to maintaining mitochondrial function and thus significantly influences cell and tissue survival.

In fact, studies done by Fernandez-Checa and Garcia-Ruiz, and others [70,71,78,251], have shown that while cells can survive near total collapse of the cytosolic GSH fraction, compromising mGSH to even a small degree, collapses $\Delta\psi_m$, sensitizes cells to oxidative insult and instigates intrinsic apoptotic and necrotic pathways. Thus, the mGSH pool constitutes a unique subcellular fraction that is vital to maintain in a narrow equilibrium for proper cell function. Despite the linkage between maintenance of mGSH and normal cell metabolism, our lab and others have shown that mGSH decreases by as much as 50% with age [252–254]. While the age-related loss of mGSH may be attributable to a variety of factors such as changes in membrane fluidity, decreased transport, or greater GSH utilization due to an increasingly oxidant rich environment, our lab has shown that GSH synthesis declines with age, which may also significantly alter mGSH levels [56]. In this regard, we showed that declines in Nrf2-dependent expression of GSH synthesis enzymes limit the basal GSH synthesis capacity. Moreover, as shown in Chapter 3, this decline in steady-state GSH levels sensitized old rat hepatocytes to acute challenge by menadione, a redox cycling agent [128]. Specifically, decreased basal levels of GSH and the GSH-dependent lipid peroxidase, GPx4, led to an increased rate and magnitude of lipid peroxidation accumulation and a concomitant loss in cell viability. Furthermore, the importance of GSH-dependent detoxification was demonstrated by the ability to remediate this increased vulnerability by providing substrates for GSH synthesis (N-acetyl cysteine). While that study demonstrated that increased

hepatocellular susceptibility to menadione was through age-related losses of GSH per se, it did not investigate the role of the distinct mGSH pool in contributing to the hepatic vulnerability to redox cycling agents. To fill this important gap in knowledge, the objective of the present work is to determine whether age-related deficits in mGSH contribute to vulnerability to menadione-mediated insults. Based on this objective, we hypothesize that age-related increases in vulnerability to an acute redox cycling challenge are due to a failure of mGSH capacity to maintain mitochondrial ETC function.

In order to test this hypothesis, we utilized acute menadione challenge in hepatocytes isolated from young and old male F344 rats and evaluated age-related differences in measures of mitochondrial function. In addition to the aforementioned increased vulnerability with age, other studies have shown that menadione has been demonstrated to directly interact with complex I in the mitochondria, deplete mGSH, decrease $\Delta\psi_m$, and induce formation of the MPTP [219,220,255]. As such, menadione is an appropriate model toxin to explore the role of age-related decline in mGSH and loss of mitochondrial function in reduced capacity to respond to acute redox cycling challenge. Herein, we show that age-related loss in mGSH correlates with increased mitochondrial vulnerability to acute menadione challenge via loss of ETC Complex I activity, a concomitant loss in basal respiration, and respiratory reserve capacity, and finally, collapse of $\Delta\psi_m$.

4.3 Methods and Materials

4.3.1 Reagents

Mitochondrial respiration medium (MiR05) was prepared as described by Gnaiger et al. on the www.bioblast.at/index.php/MiPNet14.13_Medium-MiR06 website as of January 2016. In brief, MiR05, pH 7.1, contains 0.5 mM EGTA, 3 mM MgCl₂-6H₂O, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM D-Sucrose, and 1g/L BSA (essentially fatty acid free). Collagenase, type IV, was purchased from Worthington Biochemical Corporation (Lakewood, NJ). All other chemicals used for this work were purchased from commercial sources at analytical grade.

4.3.2 Animals

Both young (4 – 6 months) and old (24 – 28 months) male F344 rats were from the National Institute on Aging animal colonies. The rats were housed in the Linus Pauling Institute animal facility and allowed to acclimatize for at least 1 week prior to any experimentation. Animals were maintained on a 12 h light cycle (7am to 7pm) and fed standard chow *ad libitum*. All animal work was approved and in accordance to IACUC guidelines (Assurance Number: A3229-

01). The AAALAC-accredited Laboratory Animal Resources Center (LARC) provided management and veterinary care.

4.3.3 Hepatocyte Isolation

Hepatocyte isolation was performed as described previously [120]. Briefly, after anesthesia via IACUC-approved protocols, the liver was perfused via a cannula in the portal vein with Hank's balanced salt solution, pH 7.4. Following removal of blood, liver cells were disassociated using collagenase solution (1 mg/ml). The resultant cell suspension was filtered through sterile gauze to remove connective tissue and debris. Parenchymal cells were isolated using gravity filtration and washed three times with Krebs-Henseleit solution, pH 7.4. Cells were resuspended in Krebs-Henseleit solution and placed in a round bottom flask and rotated at room temperature for 1 h before cell count and viability were assessed using trypan blue exclusion.

4.3.4 Analysis of Hepatocyte Mitochondrial Oxygen Consumption

Oxygen consumption rates of young and old rat hepatocytes were determined using an Oxygraph-2k High Resolution Respirometer (Oroboros®, Austria) at 25°C. Isolated hepatocytes were diluted in MiR05 medium to a cellular density of 0.5×10^6 cells/mL before being added to the instrument chamber. Following

assessment of basal respiration, hepatocellular respiratory capacity was measured by successive 0.05 μM additions of the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Additionally, electron flux through various portions of the electron transport chain was determined. Complex I activity was measured by addition of 10 mM glutamate, 2 mM malate, and 2 mM ADP. Complex I and II activity was then measured by addition of 5 mM succinate. Further differentiation of Complex I and II activity was ascertained by addition of 0.5 μM rotenone. Electron transport chain coupling and proton leak respiration were determined by addition of 2.5 μM oligomycin. Non mitochondrial respiration was measured after addition of 2.5 μM antimycin A.

4.3.5 Digitonin fractionation

In order to allow direct mitochondrial examination by controlling substrate provision while avoiding issues of mitochondrial isolation such as yield, quality, and subpopulation selection, we chose to use digitonin permeabilized primary hepatocytes as first described by Andersson and Jones [256], and later refined by Hagen, T.M. and Ames, B. [257].

Appropriate digitonin concentrations for young and old hepatocytes were determined based on successful permeabilization of the plasma membrane while mitochondrial membranes remained intact. Young and old rat hepatocytes were

treated with a digitonin concentration gradient (0.01 – 1.0 mg/10⁶ cells) over a time course (1 – 10 minutes). Permeabilization of the plasma membrane was confirmed via lack of trypan blue exclusion (>90% of cells are blue). Cells were then pelleted and glutamate dehydrogenase (GDH) activity was measured in the supernatant and pellet fractions. If mitochondria membranes were permeabilized, there was significant GDH activity in both the supernatant and pellet fractions. Mitochondria were considered to be intact and functional when >90% of total GDH activity was in the pellet and the mitochondrial respiratory control ratio (RCR), a useful general measure of mitochondrial coupling and function, was above 5. Concentrations of digitonin that met these criteria varied slightly between young and old rat hepatocytes (0.1 and 0.08 mg/10⁶ cells respectively). Time of treatment had little effect on plasma membrane permeabilization and only affected mitochondrial membranes at higher concentrations (> 0.2 mg/10⁶ cells). This digitonin:cell ratio remained effective over varying cellular concentrations tested (0.25 x 10⁶ – 1 x 10⁷ cells/mL).

4.3.6 Analysis of Mitochondrial Membrane Potential in Permeabilized Hepatocytes

Oxygen consumption rates and $\Delta\psi_m$ were simultaneously determined using an Oxygraph-2k High Resolution Respirometer (Oroboros®, Austria) at 25°C and a potential-sensitive tetraphenylphosphonium (TPP⁺) electrode. Permeabilized cells in MiR05 medium were added to the chamber and state I or substrate

starved respiration was ascertained. State IV respiration and $\Delta\psi_m$ were then measured upon the addition of 10 mM glutamate, and 2 mM malate. State III respiration and $\Delta\psi_m$ were determined by addition of 2 mM ADP. A TPP⁺ standard curve was created for each experiment by adding tetraphenylphosphonium chloride at concentrations of 0.5, 0.1, 1.5, 2, 2.5, and 3 μ M prior to the addition of permeabilized hepatocytes to the chamber.

4.3.7 Glutathione Analysis

Glutathione (GSH) content of permeabilized cells was determined according to the methods of Fariss and Reed [207] as modified by Smith et al. [56]. Briefly, suspensions were homogenized in an equal volume of 10% (w/v) perchloric acid (PCA) containing 10 mM EDTA. After deproteinization, 200 μ L of the supernatant containing internal standard (γ -glutamyl glutamate) was mixed with 50 μ L of 100 mM iodoacetic acid and the pH was adjusted to 9.0 ± 0.2 by using KOH- $K_2B_4O_7$ buffer (1 M KOH : 1.6 M $K_2B_4O_7$). Samples were placed in the dark, at room temperature for 1 h. The resulting S-carboxymethyl derivatives were subjected to further chemical modification by addition of an equal volume of dansyl-chloride solution (20 mg/mL in acetone). The solution was incubated overnight in the dark and at room temperature. The next day, 500 μ L of chloroform was added, samples were vortexed and then allowed to incubate for 1 hour in the dark, at room temperature. Samples were then centrifuged at

15,000 x g for 15 mins at 4 °C. Samples (20 µL) were separated by HPLC using a Thermo Scientific (Eugene, OR) APS-2 Hypersil column and detected on a Shimadzu (Kyoto, Japan) SPD-10AVP fluorometric detector set at 335 nm for excitation and 515 nm emission. Quantitation was obtained by integration relative to GSH and GSSG standards.

4.3.8 Statistical Analysis

All statistical analyses were performed using Excel (Microsoft, Inc.) and Prism 6 and 7 (GraphPad Software, Inc.). Error bars represent standard error of the mean. For comparisons between two samples, two-sided Student's t-test was used. Differences between samples that resulted in a p-value of ≤ 0.05 were considered statistically significant. Statistical analyses for multiple comparisons were evaluated by Student's t-test with the Sidak-Bonferroni post-hoc method.

4.4 Results

4.4.1 Acute Menadione Challenge Causes Rapid Mitochondrial Glutathione Loss

In a prior study, we established that hepatocytes from old rats were nearly twice as susceptible to menadione (LC₅₀ young: 405 µM; old: 275 µM) with significant differences in GSH loss and increased markers of lipid peroxidation within

minutes. To determine whether the enhanced toxicity from menadione insult was mediated at least in part through mitochondrial dysfunction, we treated hepatocytes isolated from young and old F344 rats with 300 μ M menadione (the \sim LC₅₀ for old) and assayed mGSH levels both initially and at 10 minutes, the fastest time point feasible to measure after menadione addition and digitonin permeabilization. With aging, initial steady-state levels of mGSH in old were nearly half that for young (Figure 1A, 36 and 68 nmol GSH/mg protein respectively). Menadione challenge caused a marked loss of mGSH in both young and old rat hepatocytes. While digitonin permeabilized cells from both age groups displayed a similar rate of mGSH loss (\sim 1.6 vs.1.8 nmol GSH/mg protein), the differences in starting levels ultimately resulted in a 50% loss of mGSH content in old rat hepatocytes within 10 minutes post menadione exposure. In contrast, hepatocytes from young rats declined by only 28% within that same timeframe. These results indicate that menadione induces a similar rate of mGSH decline regardless of age, but the magnitude of overall decline is markedly higher with age.

4.4.2 $\Delta\psi_m$ Under an Acute Menadione Challenge

As described above, mGSH depletion would be expected to acutely affect mitochondrial bioenergetics and lead to cell death [170,243,258,259]. To discern if the degree of mGSH decline evident in the aged rat hepatocytes resulted in an

exaggerated decline in mitochondrial function, we examined $\Delta\psi_m$ as a relevant parameter of overall mitochondrial function as it is the major component of the proton motive force. Using a TPP-electrode, our data show that digitonin permeabilized hepatocytes from aged rats display a marked loss of basal $\Delta\psi_m$ (21% : 31 mV) versus young. This indicates a general decline in key driving forces for ATP production and correlates with lower mGSH values with age. Menadione challenge caused a dramatic collapse in old but not young rat hepatocytes (Figure 3). Within five minutes of menadione challenge, $\Delta\psi_m$ in young hepatocytes was not significantly lower while $\Delta\psi_m$ in old rat hepatocytes has declined by 27 mV and passed the threshold for mitochondrial permeability transition pore opening (~ 100 mV). Sixty minutes after menadione challenge, $\Delta\psi_m$ in young rat hepatocytes had returned to initial values while $\Delta\psi_m$ in old rat hepatocytes remained depressed. These results demonstrate a striking difference in menadione treatment on $\Delta\psi_m$ with age both in the magnitude of membrane potential collapse and in the age-related inability to return $\Delta\psi_m$ to initial levels.

4.4.3 Mitochondrial Basal Respiration and Respiratory Reserve Capacity Under Menadione Challenge

Having established that age exacerbates menadione's effects on $\Delta\psi_m$, we hypothesized that this should translate to severe decrements in mitochondrial

oxygen consumption characteristics. A careful examination of oxygen consumption in young and old rat hepatocytes in the presence and absence of various substrates was undertaken (Figure 3A). Results showed that basal oxygen consumption was severely affected both by age and in hepatocytes from old rats challenged with menadione (Figure 3B: 34%, N = 4, $p < 0.05$). This suggests that overall ATP turnover declines with age and collapses under menadione. For the latter result, this indicates that menadione severely attenuates the cellular capacity to generate ATP. This interpretation is bolstered by results shown in Figure 3C, which indicate maximal OCR, a measure of mitochondrial elasticity to respond to energy challenges, declines dramatically with menadione in old rat hepatocytes (72%, N = 4, $p < 0.05$) but not in young. Based on these results we conclude that mitochondria lose the capacity to respond to a menadione challenge with age, which leads to a rapid deficit in cell bioenergetics.

4.4.4 Acute Menadione Challenge Significantly Effects ETC Complexes I and II in Old Rat Hepatocytes

In order to correlate the age-dependent loss in mitochondrial resiliency to menadione challenge with the severe declines in mGSH also evident with menadione, we monitored the activities of ETC components in young and old hepatocytes with and without menadione challenge. This analysis was

undertaken because of the acknowledged sensitivity of critical cysteine residues subject to glutathionylation, especially on complex I. Results from examining oxygen consumption in the presence of complex I substrates showed that there are significant age-related differences in menadione effects on mitochondria. Hepatocytes from young and old rats were treated with 300 μ M menadione for 15 minutes and then assayed for measures of mitochondrial function. In young rat hepatocytes, menadione treatment had no effect on the percent contribution of mitochondrial leak, complex I, or complex II activities to total oxygen consumption rates. However, in hepatocytes from old rats, the contributions of all of these measurements were significantly different. Leak and Complex II contributions were increased by 4.2 and 3.6-fold respectively, while Complex I activity decreased by 4.8-fold ($N = 4$, $p < 0.05$). These findings suggest that there is an age-related susceptibility to complex I inhibition from redox cycling oxidative stress, and that there is a potentially compensatory, but insufficient increase in complex II flux.

4.5 Discussion

The aging process leads to declines cellular function and resilience. Our data points to loss of GSH as a major factor in the age-related increase in vulnerability to redox cycling oxidative stressors. While GSH is independently maintained in many cellular compartments, mitochondrial glutathione is not in equilibrium

with GSH in the cytosolic compartment. We and others have observed that the age-related decline in mGSH is more severe than in other cellular compartments such as the cytosol [260] and the endoplasmic reticulum [253]. Moreover, the age-related deficits in mGSH may have more severe consequences to cell survival because it has been demonstrated that while loss of cytosolic GSH can be compensated for with other antioxidants during an oxidative stress, mGSH is critical to cellular survival [70]. While this work does not prove a causal relationship between the age-related decline in mGSH and sensitivity to menadione, it does strengthen the correlation between the loss of GSH-dependent measures of function and resilience to menadione challenge. Other important potential drivers of age-related mitochondrial dysfunction warrant further exploration such as somatic mitochondrial DNA mutations, and a pro-oxidant shift in important redox couples such as NADPH/NADP⁺, reduced and oxidized thioredoxins (Trx/TrxSS), and Cysteine/Cystine. While these and other possible factors cannot be ignored, they are beyond the scope of this examination of the role of age-related loss of GSH and GSH-dependent detoxification mechanisms in mitochondrial sensitization to acute redox cycling challenge.

The current study focused on susceptibility to mitochondrial dysfunction during acute menadione insult in freshly isolated intact and permeabilized hepatocytes from young and old rats. This cell model is appropriate for understanding aging

differences in mitochondrial dysfunction because F344 rat hepatocytes reflect the age-related losses of GSH, mGSH, and mitochondrial function that are seen in humans [127,233,261]. The use of permeabilized cells offers many of the advantages of isolated mitochondria and intact cells without suffering from the disadvantages inherent to each of those methods. For example, permeabilized cells maintain the mitochondrial network structure, and cellular compartment interactions seen in intact cell models. Additionally, they allow for the analysis of mitochondrial effects within minutes of treating cells. It also offers the direct control of ETC substrate composition and concentrations like in isolated mitochondria, but avoids mitochondrial damage and selection that often occur during the isolation [262].

We used menadione in the current study because the mechanisms of its toxicity and detoxification have been thoroughly explored [219,224]. Menadione is a potent redox cycling compound that generates a strong, persistent oxidative stress via the initial generation of superoxide anion radical followed by its dismutation into more deleterious ROS and RNS. We have previously demonstrated that the age-related increase in vulnerability to acute challenge with this compound is GSH-dependent (see Chapter 3). Additionally, menadione treatment in isolated mitochondria was previously demonstrated to deplete GSH and inhibit complex I activity [69,220]. However, prior to this report, we are not aware of any studies showing susceptibility of mitochondria to menadione

challenge as a function of age in intact cells. This distinction is important because it allows us to differentiate age-related effects of detoxification mechanisms in the cytosolic and mitochondrial compartments. For example, NqO1, the principal detoxifier of the menadione parent compound, is almost entirely localized to the cytosol, and has been shown to have 4- and 9-fold higher protein and activity levels in aged rat hepatocytes [128,263]. In contrast, GPx4 is largely found in mitochondria, and its protein levels decline by 70% in aged rat hepatocytes [116,264,265]. Thus, treatment of intact cells followed by rapid permeabilization and analysis of mitochondrial function gives us a more complete picture into the relative importance of cytosolic versus mitochondrial toxicity and detoxification changes with age.

Loss of mGSH happens almost immediately upon menadione challenge in both young and old hepatocytes. However, menadione-induced mGSH loss in young was relatively limited and remained above the baseline level of mGSH quantified in old rat. This is a critical observation because the severe magnitude of mGSH loss in aged rat cells precedes severe declines in mitochondrial membrane potential, loss of respiratory capacity and the inhibition of complex I flux. This suggests that the lower basal level of mGSH plays a critical role in the age-related loss of detoxification capacity under an acute redox cycling challenge. While a causal relationship was not determined in this work, mGSH involvement in each of these markers of mitochondrial function is well established [70,262,266].

This work clearly demonstrates that there is an age-related increase in mitochondrial susceptibility to acute redox cycling challenge and that complex I in particular is more vulnerable to inhibition. Previous studies have demonstrated an age-related shift toward a more oxidized mitochondrial environment [103,175]. This includes an increase in the GSSG content and concomitant glutathionylation of mitochondrial proteins. Reversible glutathionylation of critical cysteine residues on the 51 and 75 kDa subunits of complex I have been shown to occur when mGSH is depleted during increased oxidizing conditions in the mitochondria [91,94,95,99]. Creation of this mixed disulfide could potentially account for the basal loss of complex I activity, basal respiration, and $\Delta\psi_m$ with age [267–269]. Additionally, our lab showed that activity of glutaredoxin 2 (Grx2), the mitochondrial enzyme that de-glutathionylates redox active sulfhydryls (like those on complex I), decreases with age in rat cardiac mitochondria [254] and in cells of patients with Werner syndrome, a disease characterized by premature aging [270]. Combined with our previous study, demonstrating that GSH maintenance alone can remediate the loss of resilience seen with aging, and the established importance of mGSH regulation of complex I activity, the evidence strongly suggests that age-related loss of mGSH plays a critical role in the demonstrated mitochondrial sensitization. Therefore, these results provide a plausible mechanism that links

mGSH redox perturbations during aging and menadione insult, and the significant decline in complex I activity.

In order to further explore the causal nature of mGSH loss and mitochondrial sensitization, we are currently investigating the age-related differences in oxidative modification of complex I critical thiols during menadione challenge. Additionally, we are utilizing a newly patented triphenylphosphonium-GSH compound to titrate delivery of GSH to the mitochondria. This, in combination with compounds that specifically deplete mGSH, will allow us to elucidate whether age-related loss in mGSH is the causal factor in mitochondrial sensitization to acute redox cycling challenge.

4.6 Figures

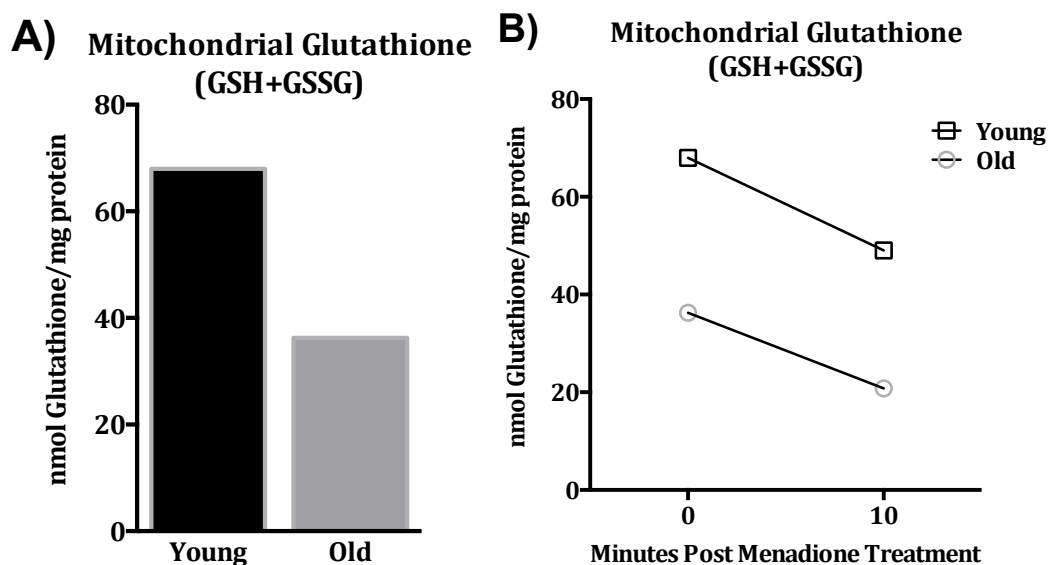


Figure 4.1. Menadione treatment causes rapid GSH loss in mitochondria from both young and old rat hepatocytes. A) Mitochondrial GSH markedly declines with age (47%, N = 1). B) The rate of menadione-mediated loss of mGSH is similar in young and old rat hepatocytes when challenged with 300 μ M menadione. However, after 10 minutes of menadione challenge, mGSH levels in young rat hepatocytes are 2.3-fold higher than in old rat hepatocytes and remain above initial mGSH levels in old rat hepatocytes (N = 1).

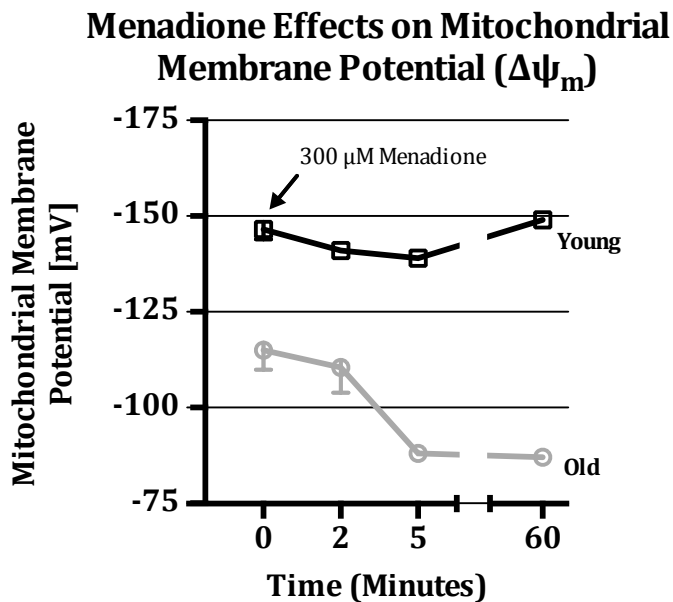
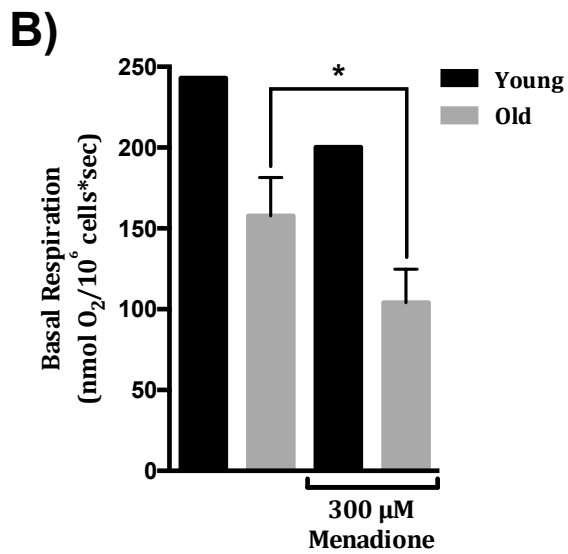
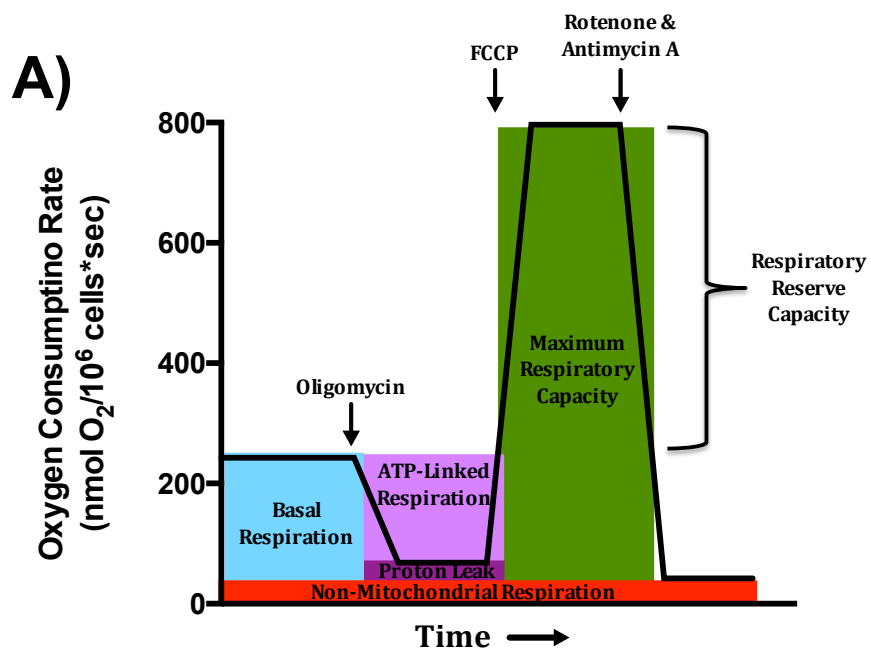


Figure 4.2. Menadione (300 μM) challenge results in the collapse of the mitochondrial membrane potential only in old rat hepatocytes. There is a 21% loss of initial $\Delta\Psi_m$ (31 ± 2.5 mV, $N = 2$) with age. Within five minutes of menadione challenge, $\Delta\Psi_m$ in young rat hepatocytes has not significantly declined while $\Delta\Psi_m$ in old rat hepatocytes has collapsed (declined by 27 ± 1.5 mV, $N = 2$), which is below the threshold for mitochondrial permeability transition pore opening (-120 - 100 mV) [262,271–273]. One hour after menadione challenge, $\Delta\Psi_m$ in young rat hepatocytes has recovered to its baseline while $\Delta\Psi_m$ in old rat hepatocytes remains attenuated.



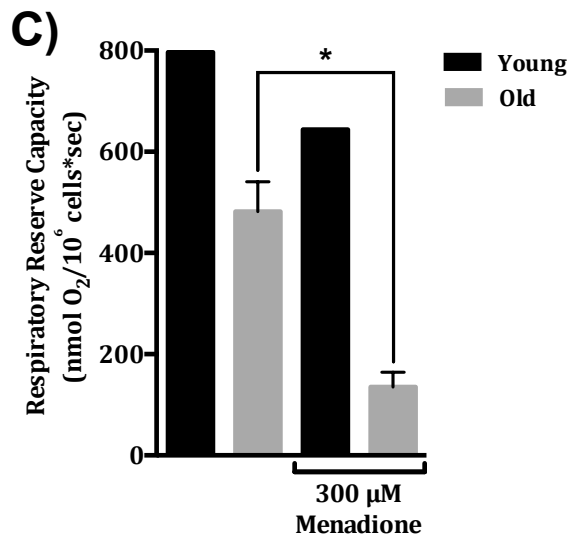


Figure 4.3. There is an age-related increase in menadione-induced loss of respiratory reserve capacity. A) Representative oxygen consumption profile of hepatocytes taken from a young rat (5 month old) highlighting the different substrate and inhibitor controlled oxygen consumption states. B&C) Treatment of old rat hepatocytes with 300 μ M menadione significantly reduces basal respiration and respiratory reserve capacity (34% and 72% respectively, N = 4, $p < 0.05$), while young rat hepatocytes are not significantly affected.

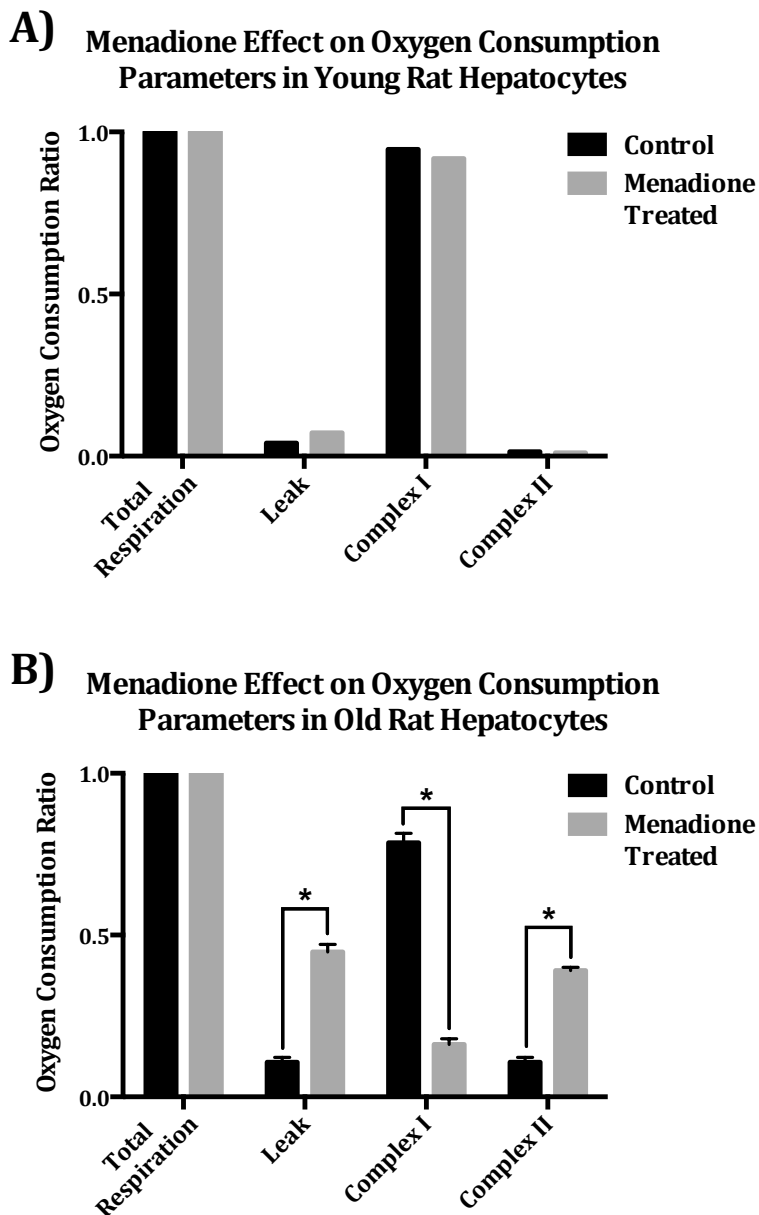


Figure 4.4. Menadione treatment (300 μ M) causes marked age-related differences in oxygen consumption parameters. In young rat hepatocytes, menadione treatment had no effect on mitochondrial leak, complex I, or complex II contributions to total oxygen consumption (4A). However, in hepatocytes from old rats, oxygen consumption contributions for all of these measurements were significantly different. Leak and Complex II contributions were increased by 4.2 and 3.6-fold respectively, and Complex I contribution decreased by 4.8-fold (N = 4, $p < 0.05$).

Chapter 5

Conclusions

Nicholas Thomas

Improvements in healthcare, sanitation, and medicine have led to the near doubling of the average lifespan in the United States over the last century [274]. The number of Americans over the age of 65 is projected to account for roughly one quarter of the US population by 2060 [1]. While this unprecedented shift in population demographics represents great strides for humanity, it is not without cost. One consequence of longer life is the increased accrual of age-associated diseases and chronic pathophysiological conditions. This is evident in the fact that over 80% of Americans over the age of 65 have at least one protracted medical condition, and many are contending with multiple chronic diseases in the later years of life [275]. As age itself is the leading risk factor for many of these pathophysiologies, this “age-wave” presages increasingly heavy economic burdens. As much as 75% of healthcare costs in the United States are already spent on the elderly, and as this demographic continues to grow, the healthcare cost implications are overwhelming.

Many age-related diseases (e.g. cardiovascular diseases, atherosclerosis, cancers, diabetes, obstructive pulmonary disorders, and a variety of neurodegenerative diseases) are linked to a decline in cellular detoxification capacity and an increased susceptibility to endogenous and exogenous oxidative stressors. In order to prevent or delay the onset of these age-related morbidities, we must understand the molecular and cellular factors that are changing with age, and how they lead to enhanced vulnerability. Thus, defining “health assurance”

biochemical pathways will allow us to develop novel strategies to lengthen average "healthspan" or the time lived free of overt diseases that limit activities of daily living.

Therefore, in order to ascertain the age-related changes that lead to a decline in detoxification capacity and increased vulnerability to a variety of toxicological challenges, this dissertation investigated the causes and consequences of the age-related loss in basal hepatic glutathione-dependent defenses, and possible methods of remediating or mitigating these declines. Herein we show that:

- Translation of Nrf2, a principal transcriptional regulator of multiple stress response pathways, is inhibited with age.
- MicroRNA-146a, an inflammation-induced microRNA, increases with age and inhibits translation of Nrf2 mRNA into protein.
- There is an enhanced age-related vulnerability to acute toxicological challenge.
- Age-related loss of GSH-dependent detoxification mechanisms plays a critical role in this vulnerability and that maintenance of GSH levels is sufficient for remediation.
- Mitochondrial function is also less resilient to acute toxicological challenge with age.
- Complex I of the electron transport chain in particular demonstrates enhanced inhibition upon acute toxicological challenge.

Thus, we have assembled a comprehensive body of research that pinpoints the GSH-dependent detoxification systems as critical “health assurance” biochemical pathways for aging. Furthermore, we have identified the mechanisms responsible for their age-related decline, the consequences of these losses in regards to acute toxicological challenge, and we have explored possible methods to remediate the resultant decrease in detoxification capacity. What follows is a more in-depth discussion of the findings and implications of this dissertation.

GSH and GSH-dependent detoxification enzymes have a wide-ranging influence on many cellular mechanisms. These include antioxidant activity, enzyme regulation, cell cycle regulation, and functioning as a substrate or cofactor for various detoxification enzymes. GSH itself as well as many GSH-dependent enzymes decline with age (Chapter 3). While all of the potential consequences of the age-related losses of GSH and GSH-dependent functions have not been fully explored, genetic ablation in model organisms has provided some insight. Mice lacking GSH synthesis genes or genes for GSH-dependent enzymes (such as GPx4) are not viable, and partial knockdown reduces average lifespan, earlier onset of and more rapid progression toward some age-related diseases, and enhanced vulnerability to xenobiotics [37]. Thus we propose that the age-related loss of GSH would result in similar characteristics and as such, GSH and GSH-dependent detoxification enzymes responsible for maintaining cellular homeostasis and function are excellent candidates for “health assurance”

pathways. In support of this, many age-related diseases present with decreased GSH levels or GSH-dependent enzyme function, and data from our lab and others has demonstrated that pharmaceutical repletion of GSH can enhance resilience to toxicological challenge [130]. Despite the prevalence of research highlighting the importance of GSH in cellular homeostasis, the causes of consequences of its age-related loss have still not been fully elucidated.

Previous work in our lab has demonstrated that an age-related loss of Nrf2, the chief transcriptional regulator of GSH synthesis enzymes, leads to a concomitant decline in cellular GSH. We also showed that this decline was occurring at the proteostatic level, as Nrf2 messenger RNA was unchanged with age. However, the mechanism(s) responsible for Nrf2 protein loss at a time when an increasingly pro-oxidant cellular environment should dictate Nrf2 induction remained undefined. In this dissertation, we investigated the age-related mechanisms that lead to the loss of Nrf2 and the subsequent loss of GSH-dependent detoxification pathways. Our findings demonstrated that in hepatocytes isolated from old rats, attenuated translation is the cause for the loss of Nrf2 protein levels (Chapter 2). While a general decline in protein synthesis has been shown in aging, Nrf2 loss is more dramatic, with a 40% decline.

In further exploring the precise mechanism(s) of Nrf2 loss with age, we found a role for microRNAs (miRs) that arise during pro-inflammatory conditions. We show that miR-146a reduces Nrf2 translation directly or indirectly due to its own increase with age. miR-146a is one of a cadre of inflammation-induced miRs that we found to increase with age [162]. While these miRs are typically induced in an acute fashion and help resolve an inflammatory response, the chronically inflamed environment in the aging rat liver may be creating a detrimental cycle of Nrf2 repression, and a concomitant loss of detoxification capacity, which leads to an inability to resolve the inflammation and terminate the cycle.

Thus, we have determined that it is an age-related inhibition of Nrf2 translation by an inflammation-induced miR that leads to decreased transcription of GSH and GSH-dependent detoxification genes. While technical limitations prevented us from determining whether a biochemical inhibition of miR-146a could remediate Nrf2 levels, over expression of Nrf2 even in age-rat hepatocytes was sufficient to restore GSH synthesis genes. This implies that GSH and GSH-dependent pathways could be restored during aging if Nrf2 levels are maintained. However, it cannot be ruled out that Nrf2 repression is a compensatory mechanism to reduce the risk of developing cancers. Nrf2 regulates a myriad of cellular detoxification and xenobiotic response genes that result in cytoprotection and resistance to apoptosis [168]. In support of this, increased Nrf2 levels have been implicated in the progression of some cancers

[140]. Thus pharmacological methods to increase Nrf2 levels could conceivably do more harm than good.

Nevertheless, it is clear that loss of Nrf2 results in a decline in many cell survival mechanisms including the GSH-dependent detoxification pathways. The extent to which Nrf2 loss results in an age-related decline in xenobiotic and oxidant-induced stress response remains to be elucidated. Numerous studies have demonstrated an age-related increase in vulnerability to drugs that are detoxified in an Nrf2-dependent method, which supports its pivotal role in protection against a variety of xenobiotic insults. However, definitive examinations into the extent that Nrf2 loss influences toxicological resilience thresholds and which Nrf2-mediated detoxification pathways are most critical remain scant.

In order to further define which Nrf2-mediated detoxification mechanisms could be considered “health assurance” pathways, we utilized menadione, a model redox cycling compound, which is detoxified by several Nrf2-dependent mechanisms (Chapter 3). We found that menadione was significantly more toxic in old rat hepatocytes at nearly every concentration used. The aging process leads to disparate changes in hepatic detoxification capacity even within Nrf2-regulated genes. For example, many phase I enzymes are unaffected by age, while phase II detoxification processes are more variable [214–216]. Therefore,

while a general age-related loss in basal detoxification capacity is apparent, further examination was performed to identify if certain pathways play a more important role in the age-related increase in toxicological vulnerability than others.

We found that although there were compensations in detoxification mechanisms associated with limiting the production of pro-oxidant species (e.g. decrease in redox cycling activation enzyme CPR and increase in quinone reducing enzyme NQO1), these changes were insufficient to protect the cell from the loss of GSH and the GSH-dependent enzyme GPx4. This suggests that cellular defenses with aging may be more geared toward attempting to limit the initiation of oxidative damage due to a loss in the capacity to detoxify and repair the damage once it occurs. This could conceivably be an effective adaptation to the age-related shift toward a pro-oxidant cellular environment with age. However, our findings clearly show that it cannot protect cells from an acute toxicological challenge.

While our data definitively point to losses in GSH and GSH utilizing enzymes as the crux of this age-related sensitivity, they do not discern at what point limiting GSH becomes problematic. We and others have previously shown that while total hepatic GSH declines by as much 30% with age in the rat liver [56,131,134,232–234], it remains at millimolar levels even in very old animals. Although a GSH K_m value for GPx4 has not yet been determined due to the

technical difficulty of producing and purifying the enzyme [276], GSH K_m values for other GPxs have been measured between 0.2 – 2.8 mM in the rat liver [277–280]. Thus, even though GSH concentrations are quite high in young rat livers (between 5 – 10mM), the significant age-related decline could limit the effectiveness of GPx-dependent detoxification.

It was not known prior to this work whether attenuated GSH concentrations in the liver make it more susceptible to menadione insult. Menadione causes a rapid loss of GSH in hepatocytes from both young and old rats, but the rate and degree of GSH loss is more extensive in older animals. Additionally, the ability to eliminate the age-related vulnerability to acute menadione challenge by providing GSH synthesis substrates buttresses the notion that GSH and GSH-dependent detoxification represent the critical “health assurance” pathways against acute redox cycling challenges, and suggests a larger susceptibility to a variety of other toxins, oxidants, and environmental mutagens. Another interesting factor revealed by this work is the effectiveness of preventative GSH maintenance as a protectant against acute oxidative challenge. This is suggestive of the possibility to utilize NAC or other GSH enhancing pharmaceuticals in a prophylactic capacity. Potential uses range from taking NAC before expected incidence of acute challenge, such as when traveling to smog filled cities, or before surgery with potentially harmful anesthetics, to daily supplementation. While the possibilities are intriguing, there are obvious limitations: NAC

supplementation in combination with some drugs will speed their clearance and limit their intended effect, supraphysiological amounts of NAC could lead to toxic levels of cysteine and cysteine buildup as could the inability to process the supplement due to impaired liver or kidney function, and NAC and other GSH enhancers have been shown to increase the resistance of cancers to chemotherapeutics. Nonetheless, utilization of NAC as a prophylactic bears further investigation.

An additional aspect revealed in this study is that, after the toxin, GSH levels in young rat hepatocytes never decreased below ~35% of initial levels, versus the 90% lost by old. This suggests that when the menadione-mediated oxidation crosses a critical threshold of GSH, cellular toxicity rapidly ensues. GSH is independently maintained in many cellular compartments. Mitochondrial glutathione is not in equilibrium with GSH in the cytosolic compartment and makes up about 15 - 20% of total cellular GSH. This suggests that the mitochondrial GSH pool may be better protected in hepatocytes from young animals and that this is the critical difference in age-related resilience. This idea is bolstered by the fact that mGSH loss is more severe with aging (as much as 50% decline) and that depletion of mGSH is sufficient to induce apoptotic and necrotic cell death pathways even if cytosolic GSH is maintained.

Investigation of age-related differences in acute menadione challenge on markers of mitochondrial function revealed marked differences. Loss of mGSH was immediately evident in both age groups, but remained relatively limited in young with levels never dropping to the baseline level of old. Interestingly, these results suggest that our hypothesis that the mGSH pool is being protected by the cytosolic GSH pool in young but not old is unlikely to be the case. Instead, results from both chapters 3 and 4 indicate that there is a threshold of GSH that must be maintained in order to preserve cell viability. This is supported by research showing that animal models with the modulatory subunit of the GCL enzyme knocked out demonstrate enhanced susceptibility to oxidative stress [37].

Furthermore, the effects of menadione on markers of mitochondrial function revealed that complex I inhibition is significantly enhanced in old rat hepatocytes but not in young. This led to a concomitant dissipation of the mitochondrial membrane potential only in aged rat hepatocytes. GSH is an important regulator of complex I function, primarily through reversible glutathionylation and protection of critical sulfhydryl residues during oxidative stress. While we have not currently explored the oxidation states of these residues, we hypothesize that the loss of mGSH with age will result in irreversible oxidation of these thiols rather than glutathionylation. In support of this, we found that while mitochondria in young rat hepatocytes had returned to

basal activity levels one hour after menadione challenge, mitochondrial function in old rat hepatocytes remained suppressed.

Thus, we clearly demonstrate that there is an age-related increase in mitochondrial susceptibility to acute redox cycling challenge, and that complex I in particular is more vulnerable to inhibition. This and our previous work strongly suggest that the age-related loss of GSH and mGSH play a critical role in enhancing age-related vulnerability to acute toxicological challenge. On a more speculative note, loss of GSH levels and regulation could be at least partially responsible for sensitizing cells, tissues, organs, and organisms to oxidative stress with age. In support of this notion, GSH plays an important role in maintaining redox sensitive iron-sulfur (Fe-S) complexes in critical proteins such as aconitase in the tricarboxylic acid cycle and complexes I, III, and IV in the ETC. GSH depletion results in the destabilization of these Fe-S complexes, and the release of iron from the protein, which can then participate in Fenton reactions to produce hydroxyl radicals and other highly toxic reactive oxygen species. Furthermore, loss of GSH and GSH-dependent detoxification systems could play a substantial role in the onset and progression of many age-related pathophysiologies due to the importance of GSH as a cell and mitochondrial protectant from ROS induced damage both as an oxidant scavenger as in the case of peroxynitrite and as a substrate for detoxification as seen with the GSTs, GPxs, thioredoxins, and peroxiredoxins. In order to further explore the potentially

causal nature we are suggesting, further experiments must be performed in which GSH and mGSH levels are carefully titrated in order to establish whether a critical threshold exists. Determining this threshold and developing methodologies to pharmacologically regulate GSH and mGSH levels could help delay the onset or reduce the severity of age-related diseases. While cytosolic GSH can be enhanced by providing synthesis substrates as demonstrated in Chapter 3, mGSH regulation is much more difficult. To that end, our lab is developing a newly patented triphenylphosphonium-GSH compound to titrate delivery of GSH to the mitochondria.

In summary, this dissertation has identified the GSH and GSH-dependent detoxification mechanisms to be critical pathways for maintaining age-related resilience to acute toxicological challenges. Additionally, we have determined the molecular mechanisms for the age-related loss of Nrf2, which drives the loss of these pathways. Thus we have identified multiple lesions that result in age-related vulnerability to acute oxidant challenge. This work sets the stage to establish these mechanisms as “health assurance” pathways and identify them as important pharmaceutical targets for maintaining cellular resilience and organ function with age.

Bibliography

- [1] S. Colby, J. Ortman, Projections of the Size and Composition of the U.S. Population: 2014 to 2060., 2015.
- [2] G.J. Brewer, Epigenetic oxidative redox shift (EORS) theory of aging unifies the free radical and insulin signaling theories, *Exp. Gerontol.* 45 (2010) 173–179. doi:10.1016/j.exger.2009.11.007.
- [3] C.C. Caldeira Da Silva, F.M. Cerqueira, L.F. Barbosa, M.H.G. Medeiros, A.J. Kowaltowski, Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity, *Aging Cell.* 7 (2008) 552–560. doi:10.1111/j.1474-9726.2008.00407.x.
- [4] D. Ghosh, K.R. LeVault, A.J. Barnett, G.J. Brewer, A Reversible Early Oxidized Redox State That Precedes Macromolecular ROS Damage in Aging Nontransgenic and 3xTg-AD Mouse Neurons, *J. Neurosci.* 32 (2012) 5821–5832. doi:10.1523/JNEUROSCI.6192-11.2012.
- [5] D. Ghosh, K.R. LeVault, G.J. Brewer, Relative importance of redox buffers GSH and NAD(P)H in age-related neurodegeneration and Alzheimer disease-like mouse neurons, *Aging Cell.* 13 (2014) 631–640. doi:10.1111/accel.12216.
- [6] K.K. Griendling, G.A. FitzGerald, Oxidative Stress and Cardiovascular Injury Part I: Basic Mechanisms and In Vivo Monitoring of ROS, *Circulation.* 108 (2003) 1912–1916. doi:10.1161/01.CIR.0000093660.86242.BB.
- [7] E. Ho, K. Karimi Galougahi, C.C. Liu, R. Bhindi, G.A. Figtree, Biological markers of oxidative stress: Applications to cardiovascular research and practice, *Redox Biol.* 1 (2013) 483–491. doi:10.1016/j.redox.2013.07.006.
- [8] D.P. Jones, Redefining Oxidative Stress, *Antioxid. Redox Signal.* 8 (2006) 1–16. doi:10.1089/ars.2006.8.1865.
- [9] D.P. Jones, Redox theory of aging., *Redox Biol.* 5 (2015) 71–79. doi:10.1016/j.redox.2015.03.004.
- [10] J.E. Klaunig, L.M. Kamendulis, The role of oxidative stress in carcinogenesis., *Annu. Rev. Pharmacol. Toxicol.* 44 (2004) 239–267. doi:10.1146/annurev.pharmtox.44.101802.121851.
- [11] J. Liu, W. Qu, M.B. Kadiiska, Role of oxidative stress in cadmium toxicity and carcinogenesis, *Toxicol. Appl. Pharmacol.* 238 (2009) 209–214. doi:10.1016/j.taap.2009.01.029.
- [12] C. López-Otín, M.A. Blasco, L. Partridge, M. Serrano, G. Kroemer, XThe hallmarks of aging, *Cell.* 153 (2013). doi:10.1016/j.cell.2013.05.039.
- [13] A.C. Maritim, R.A. Sanders, J.B. Watkins, Diabetes, oxidative stress, and antioxidants: A review, *J. Biochem. Mol. Toxicol.* 17 (2003) 24–38. doi:10.1002/jbt.10058.
- [14] J. Paredes, D. Jones, M. Wilson, J. Herndon, Age-related alterations of plasma glutathione and oxidation of redox potentials in chimpanzee (*Pan troglodytes*) and rhesus monkey (*Macaca mulatta*), *Age (Omaha).* (2014)

- 719–732.
- [15] J.L. Rains, S.K. Jain, Oxidative stress, insulin signaling, and diabetes, *Free Radic. Biol. Med.* 50 (2011) 567–575. doi:10.1016/j.freeradbiomed.2010.12.006.
- [16] R.V. Sekhar, S.V. McKay, S.G. Patel, A.P. Guthikonda, V.T. Reddy, A. Balasubramanyam, F. Jahoor, Glutathione synthesis is diminished in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine, *Diabetes Care.* (2011) 162–167.
- [17] D. Ziech, R. Franco, A.G. Georgakilas, S. Georgakila, V. Malamou-Mitsi, O. Schoneveld, A. Pappa, M.I. Panayiotidis, The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development, *Chem. Biol. Interact.* 188 (2010) 334–339. doi:10.1016/j.cbi.2010.07.010.
- [18] National Health Expenditure Projections 2015-2025, 2015.
- [19] R. Fahey, A. Sundquist, Evolution of glutathione metabolism., in: *Adv Enzym. Relat Areas Mol Biol*, 1991: pp. 1–53. doi:10.1002/9780470123102.ch1.
- [20] R.C. Fahey, R.M. Buschbacher, G.L. Newton, The evolution of glutathione metabolism in phototrophic microorganisms, *J. Mol. Evol.* 25 (1987) 81–88. doi:10.1007/BF02100044.
- [21] A.R. Sundquist, R.C. Fahey, The function of gamma-glutamylcysteine and bis-gamma-glutamylcystine reductase in *Halobacterium halobium.*, *J. Biol. Chem.* 264 (1989) 719–725.
- [22] A.R. Sundquist, R.C. Fahey, Evolution of antioxidant mechanisms: Thiol-Dependent peroxidases and thioltransferase among procaryotes, *J. Mol. Evol.* 29 (1989) 429–435. doi:10.1007/BF02602913.
- [23] N. Kaplowitz, The importance and regulation of hepatic glutathione, *Yale J. Biol. Med.* 54 (1981) 497–502. doi:10.1055/s-2008-1040481.
- [24] A. Meister, Metabolism and functions of glutathione, *Trends Biochem. Sci.* 6 (1981) 231–234. doi:10.1016/0968-0004(81)90084-0.
- [25] A. Meister, M.E. Anderson, Glutathione, *Annu. Rev. Biochem.* 52 (1983) 711–760. doi:10.1146/annurev.bi.52.070183.003431.
- [26] M. Anderson, Glutathione and glutathione disulfide in biological samples, *Methods Enzymol.* 113 (1985) 548–555. doi:10.1016/S0076-6879(85)13073-9.
- [27] T.M. Bray, C.G. Taylor, Tissue glutathione, nutrition, and oxidative stress., *Can. J. Physiol. Pharmacol.* 71 (1993) 746–51. doi:10.1139/y93-111.
- [28] B.M. Lomaestro, M. Malone, Glutathione in health and disease: Pharmacotherapeutic issues, *Ann. Pharmacother.* 29 (1995) 1263–1273. doi:10.1177/106002809502901213.
- [29] H. Sies, Glutathione and its role in cellular functions, *Free Radic Biol Med.* 27 (1999) 916–921. doi:http://dx.doi.org/10.1016/S0891-5849(99)00177-X.

- [30] R. Njålsson, S. Norgren, Physiological and pathological aspects of GSH metabolism., *Acta Paediatr.* 94 (2005) 132–137. doi:10.1080/08035250410025285.
- [31] O.W. Griffith, a Meister, Glutathione: interorgan translocation, turnover, and metabolism., *Proc. Natl. Acad. Sci. U. S. A.* 76 (1979) 5606–5610. doi:10.1073/pnas.76.11.5606.
- [32] Y. Kera, K.E. Penttilä, K.O. Lindros, Glutathione replenishment capacity is lower in isolated perivenous than in periportal hepatocytes., *Biochem. J.* 254 (1988) 411–7. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1135093&tool=pmcentrez&rendertype=abstract>.
- [33] R.M. Liu, J. Choi, H.J. Forman, Oxidant-induced regulation of glutathione synthesis, *Curr. Protoc. Toxicol.* Chapter 6 (2001) Unit 6 7. doi:10.1002/0471140856.tx0607s08.
- [34] N. Couto, J. Wood, J. Barber, The role of glutathione reductase and related enzymes on cellular redox homeostasis network, *Free Radic. Biol. Med.* 95 (2016) 27–42. doi:10.1016/j.freeradbiomed.2016.02.028.
- [35] S.C. Lu, Glutathione synthesis, *Biochim. Biophys. Acta.* 1830 (2013) 3143–53. doi:10.1016/j.bbagen.2012.09.008.
- [36] S.C. Lu, Regulation of glutathione synthesis, *Mol. Aspects Med.* 30 (2009) 42–59. doi:10.1016/j.mam.2008.05.005.
- [37] Y. Chen, H.G. Shertzer, S.N. Schneider, D.W. Nebert, T.P. Dalton, Glutamate cysteine ligase catalysis: Dependence on ATP and modifier subunit for regulation of tissue glutathione levels, *J. Biol. Chem.* 280 (2005) 33766–33774. doi:10.1074/jbc.M504604200.
- [38] D.M. Krzywanski, D.A. Dickinson, K.E. Iles, A.F. Wigley, C.C. Franklin, R.M. Liu, T.J. Kavanagh, H.J. Forman, Variable regulation of glutamate cysteine ligase subunit proteins affects glutathione biosynthesis in response to oxidative stress, *Arch. Biochem. Biophys.* 423 (2004) 116–125. doi:10.1016/j.abb.2003.11.004.
- [39] F. Gu, V. Chauhan, A. Chauhan, Impaired synthesis and antioxidant defense of glutathione in the cerebellum of autistic subjects: Alterations in the activities and protein expression of glutathione-related enzymes, *Free Radic. Biol. Med.* 65 (2013) 488–496. doi:10.1016/j.freeradbiomed.2013.07.021.
- [40] C.J. Harvey, R.K. Thimmulappa, A. Singh, D.J. Blake, G. Ling, N. Wakabayashi, J. Fujii, A. Myers, S. Biswal, Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress, *Free Radic. Biol. Med.* 46 (2009) 443–453. doi:10.1016/j.freeradbiomed.2008.10.040.
- [41] D.A. Dickinson, D.R. Moellering, K.E. Iles, R.P. Patel, A.L. Levonen, A. Wigley, V.M. Darley-Usmar, H.J. Forman, Cytoprotection against oxidative stress and the regulation of glutathione synthesis, *Biol. Chem.* 384 (2003) 527–537. doi:10.1515/BC.2003.061.

- [42] S.R. Soltaninassab, K.R. Sekhar, M.J. Meredith, M.L. Freeman, Multi-faceted regulation of gamma-glutamylcysteine synthetase., *J. Cell. Physiol.* 182 (2000) 163–170. doi:10.1002/(SICI)1097-4652(200002)182:2<163::AID-JCP4>3.0.CO;2-1.
- [43] R.M. Liu, H. Hu, T.W. Robison, H.J. Forman, Increased ??-Glutamylcysteine Synthetase and ??-Glutamyl Transpeptidase Activities Enhance Resistance of Rat Lung Epithelial L2 Cells to Quinone Toxicity, *Am. J. Respir. Cell Mol. Biol.* 14 (1996) 192–197.
- [44] M.M. Shi, A. Kugelman, T. Iwamoto, L. Tian, H.J. Forman, Quinone-induced Oxidative Stress Elevates Glutathione and Induces ??-Glutamylcysteine Synthetase Activity in Rat Lung Epithelial L2 Cells, *J. Biol. Chem.* 269 (1994) 26512–26517.
- [45] R. Venugopal, a K. Jaiswal, Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene., *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 14960–14965. doi:10.1073/pnas.93.25.14960.
- [46] K. Chan, X.-D. Han, Y.W. Kan, An important function of Nrf2 in combating oxidative stress: Detoxification of acetaminophen, *Proc. Natl. Acad. Sci.* 98 (2001) 4611–4616. doi:10.1073/pnas.081082098.
- [47] A. Wild, R. Mulcahy, Regulation of gamma-glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defenses., *Free Radic Res.* 32 (2000) 281–301. doi:10.1080/10715760000300291.
- [48] P. Moi, K. Chan, I. Asunis, A. Cao, Y.W. Kan, Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region., *Proc. Natl. Acad. Sci.* 91 (1994) 9926–9930. doi:10.1073/pnas.91.21.9926.
- [49] K. Itoh, T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto, Y. Nabeshima, An Nrf2/Small Maf Heterodimer Mediates the Induction of Phase II Detoxifying Enzyme Genes through Antioxidant Response Elements, *Biochem. Biophys. Res. Commun.* 236 (1997) 313–322. doi:10.1006/bbrc.1997.6943.
- [50] J.D. Hayes, M. McMahon, Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention, *Cancer Lett.* 174 (2001) 103–113. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11689285.
- [51] T.G. Belgard, A.C. Marques, P.L. Oliver, H.O. Abaan, T.M. Sirey, A. Hoerder-Suabedissen, F. García-Moreno, Z. Molnár, E.H. Margulies, C.P. Ponting, A transcriptomic atlas of mouse neocortical layers, *Neuron.* 71 (2011) 605–616. doi:10.1016/j.neuron.2011.06.039.
- [52] J.D. Hayes, A.T. Dinkova-Kostova, The Nrf2 regulatory network provides an

- interface between redox and intermediary metabolism, *Trends Biochem. Sci.* 39 (2014) 199–218. doi:10.1016/j.tibs.2014.02.002.
- [53] T. Toki, J. Itoh, J. Kitazawa, K. Arai, K. Hatakeyama, J. Akasaka, K. Igarashi, N. Nomura, M. Yokoyama, M. Yamamoto, E. Ito, Human small Maf proteins form heterodimers with CNC family transcription factors and recognize the NF-E2 motif, *Oncogene*. 14 (1997) 1901–1910. doi:10.1038/sj.onc.1201024.
- [54] T. Nguyen, H.C. Huang, C.B. Pickett, Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK, *J. Biol. Chem.* 275 (2000) 15466–15473. doi:10.1074/jbc.M000361200.
- [55] H. Motohashi, F. Katsuoka, J.D. Engel, M. Yamamoto, Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway, *Proc. Natl. Acad. Sci.* 101 (2004) 6379–6384. doi:10.1073/pnas.0305902101.
- [56] J.H. Suh, S. V Shenvi, B.M. Dixon, H. Liu, A.K. Jaiswal, R.-M. Liu, T.M. Hagen, Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid., *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 3381–6. doi:10.1073/pnas.0400282101.
- [57] S. V Shenvi, E.J. Smith, T.M. Hagen, Transcriptional regulation of rat gamma-glutamyl cysteine ligase catalytic subunit gene is mediated through a distal antioxidant response element, *Pharmacol Res.* 60 (2009) 229–236. doi:S1043-6618(09)00169-8 [pii]\r10.1016/j.phrs.2009.06.003.
- [58] K.P. Shay, A.J. Michels, W. Li, A.N.T. Kong, T.M. Hagen, Cap-independent Nrf2 translation is part of a lipoic acid-stimulated detoxification stress response, *Biochim. Biophys. Acta - Mol. Cell Res.* 1823 (2012) 1102–1109. doi:10.1016/j.bbamcr.2012.04.002.
- [59] E.J. Smith, K.P. Shay, N.O. Thomas, J.A. Butler, L.F. Finlay, T.M. Hagen, Age-related loss of hepatic Nrf2 protein homeostasis: Potential role for heightened expression of miR-146a, *Free Radic. Biol. Med.* 89 (2015) 1184–1191. doi:10.1016/j.freeradbiomed.2015.11.003.
- [60] J. Viña, R. Hems, H. a Krebs, Maintenance of glutathione content is isolated hepatocytes., *Biochem. J.* 170 (1978) 627–630.
- [61] H.R. López-Mirabal, J.R. Winther, Redox characteristics of the eukaryotic cytosol, *Biochim. Biophys. Acta - Mol. Cell Res.* 1783 (2008) 629–640. doi:10.1016/j.bbamcr.2007.10.013.
- [62] D.M. Townsend, Y. Manevich, L. He, Y. Xiong, R.R. Bowers, S. Hutchens, K.D. Tew, Nitrosative Stress – Induced S -Glutathionylation of Protein Disulfide Isomerase Leads to Activation of the Unfolded Protein Response, *Cancer Res.* 69 (2009) 7626–7634. doi:10.1158/0008-5472.CAN-09-0493.
- [63] S. Chakravarthi, N.J. Balleid, Glutathione is required to regulate the formation of native disulfide bonds within proteins entering the secretory pathway, *J. Biol. Chem.* 279 (2004) 39872–39879. doi:10.1074/jbc.M406912200.

- [64] S. Chakravarthi, C.E. Jessop, N.J. Bulleid, The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress, ©2006 Eur. Mol. Biol. Organ. EMBO Reports EMBO Reports. 7 (2006) 271–275. doi:10.1038/sj.embor.7400645.
- [65] G. Bellomo, M. Vairetti, L. Stivala, F. Mirabelli, P. Richelmi, S. Orrenius, Demonstration of nuclear compartmentalization of glutathione in hepatocytes., Proc. Natl. Acad. Sci. 89 (1992) 4412–4416. doi:10.1073/pnas.89.10.4412.
- [66] J.M. Hansen, Y.-M. Go, D.P. Jones, Nuclear and Mitochondrial Compartmentation of Oxidative Stress and Redox Signaling, Annu. Rev. Pharmacol. Toxicol. 46 (2006) 215–234. doi:10.1146/annurev.pharmtox.46.120604.141122.
- [67] J.L. García-Giménez, J. Markovic, F. Dasí, G. Queval, D. Schnaubelt, C.H. Foyer, F. V. Pallardó, Nuclear glutathione, Biochim. Biophys. Acta - Gen. Subj. 1830 (2013) 3304–3316. doi:10.1016/j.bbagen.2012.10.005.
- [68] A.R. Cardoso, B. Chausse, F.M. Da Cunha, L.A. Luévano-Martínez, T.B.M. Marazzi, P.S. Pessoa, B.B. Queliconi, A.J. Kowaltowski, Mitochondrial compartmentalization of redox processes, Free Radic. Biol. Med. 52 (2012) 2201–2208. doi:10.1016/j.freeradbiomed.2012.03.008.
- [69] M.L. Circu, C. Rodriguez, R. Maloney, M.P. Moyer, T.Y. Aw, Contribution of mitochondrial GSH transport to matrix GSH status and colonic epithelial cell apoptosis, Free Radic. Biol. Med. 44 (2008) 768–778. doi:10.1016/j.freeradbiomed.2007.09.011.
- [70] M. Marí, A. Morales, A. Colell, C. García-Ruiz, J.C. Fernández-Checa, Mitochondrial glutathione, a key survival antioxidant., Antioxid. Redox Signal. 11 (2009) 2685–2700. doi:10.1089/ars.2009.2695.
- [71] C. García-Ruiz, A. Morales, A. Colell, J. Rodés, J.R. Yi, N. Kaplowitz, J.C. Fernández-Checa, Evidence that the rat hepatic mitochondrial carrier is distinct from the sinusoidal and canalicular transporters for reduced glutathione: Expression studies in *Xenopus laevis* oocytes, J. Biol. Chem. 270 (1995) 15946–15949. doi:10.1074/jbc.270.27.15946.
- [72] V. Ribas, C. García-Ruiz, J.C. Fernández-Checa, Glutathione and mitochondria, Front. Pharmacol. 5 JUL (2014). doi:10.3389/fphar.2014.00151.
- [73] L.H. Lash, Measurement of glutathione transport, Curr Protoc Toxicol. Chapter 6 (2001) Unit6 3. doi:10.1002/0471140856.tx0603s00.
- [74] L.H. Lash, Mitochondrial glutathione transport: Physiological, pathological and toxicological implications, Chem. Biol. Interact. 163 (2006) 54–67. doi:10.1016/j.cbi.2006.03.001.
- [75] Q. Zhong, D.A. Putt, F. Xu, L.H. Lash, Hepatic mitochondrial transport of glutathione: studies in isolated rat liver mitochondria and H4IIE rat hepatoma cells, Arch Biochem Biophys. 474 (2008) 119–127. doi:10.1016/j.abb.2008.03.008.
- [76] C.K. Kamga, S.X. Zhang, Y. Wang, Dicarboxylate carrier-mediated

- glutathione transport is essential for reactive oxygen species homeostasis and normal respiration in rat brain mitochondria., *Am. J. Physiol. Cell Physiol.* 299 (2010) C497-505. doi:10.1152/ajpcell.00058.2010.
- [77] B. V Chernyak, P. Bernardi, The mitochondrial permeability transition pore is modulated by oxidative agents through both pyridine nucleotides and glutathione at two separate sites., *Eur. J. Biochem.* 238 (1996) 623–630. doi:DOI 10.1111/j.1432-1033.1996.0623w.x.
- [78] J.C. Fernandez-Checa, N. Kaplowitz, Hepatic mitochondrial glutathione: Transport and role in disease and toxicity, *Toxicol. Appl. Pharmacol.* 204 (2005) 263–273. doi:10.1016/j.taap.2004.10.001.
- [79] R.M. Lopachin, T. Gavin, Reactions of electrophiles with nucleophilic thiolate sites: relevance to pathophysiological mechanisms and remediation, *Free Radic. Res.* 50 (2016) 195–205. doi:10.3109/10715762.2015.1094184doi.org/10.3109/10715762.2015.1094184.
- [80] L.B. Poole, The basics of thiols and cysteines in redox biology and chemistry, *Free Radic. Biol. Med.* 80 (2015) 148–157. doi:10.1016/j.freeradbiomed.2014.11.013.
- [81] S.G. Tajc, B.S. Tolbert, R. Basavappa, B.L. Miller, Direct determination of thiol pKa by isothermal titration microcalorimetry, *J. Am. Chem. Soc.* 126 (2004) 10508–10509. doi:10.1021/ja047929u.
- [82] K. Aquilano, S. Baldelli, M.R. Ciriolo, Glutathione: New roles in redox signalling for an old antioxidant, *Front. Pharmacol.* 5 AUG (2014). doi:10.3389/fphar.2014.00196.
- [83] F.Q. Schafer, G.R. Buettner, Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple, *Free Radic. Biol. Med.* 30 (2001) 1191–1212. doi:10.1016/S0891-5849(01)00480-4.
- [84] D.P. Jones, Redox potential of GSH/GSSG couple: Assay and biological significance, *Methods Enzymol.* 348 (2002) 93–112. doi:10.1016/S0076-6879(02)48630-2.
- [85] D.P. Jones, Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control, *FASEB J.* (2004). doi:10.1096/fj.03-0971fje.
- [86] R.D. DeLaune, K.R. Reddy, REDOX POTENTIAL, in: *Encycl. Soils Environ.*, 2005: pp. 366–371. doi:10.1016/B0-12-348530-4/00212-5.
- [87] Z. Cheng, L.D. Arscott, D.P. Ballou, C.H. Williams, The relationship of the redox potentials of thioredoxin and thioredoxin reductase from *Drosophila melanogaster* to the enzymatic mechanism: Reduced thioredoxin is the reductant of glutathione in *Drosophila*, *Biochemistry.* 46 (2007) 7875–7885. doi:10.1021/bi700442r.
- [88] D.P. Jones, Redox sensing: Orthogonal control in cell cycle and apoptosis signalling, in: *J. Intern. Med.*, 2010: pp. 432–448. doi:10.1111/j.1365-2796.2010.02268.x.

- [89] Y.M. Go, D.P. Jones, Cysteine/cystine redox signaling in cardiovascular disease, *Free Radic. Biol. Med.* 50 (2011) 495–509. doi:10.1016/j.freeradbiomed.2010.11.029.
- [90] J. Chiu, I.W. Dawes, Redox control of cell proliferation, *Trends Cell Biol.* 22 (2012) 592–601. doi:10.1016/j.tcb.2012.08.002.
- [91] L. Michelet, M. Zaffagnini, V. Massot, E. Keryer, H. Vanacker, M. Miginiac-Maslow, E. Issakidis-Bourguet, S.D. Lemaire, Thioredoxins, glutaredoxins, and glutathionylation: New crosstalks to explore, *Photosynth. Res.* 89 (2006) 225–245. doi:10.1007/s11120-006-9096-2.
- [92] J. Garcia, D. Han, H. Sancheti, L.P. Yap, N. Kaplowitz, E. Cadenas, Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates, *J. Biol. Chem.* 285 (2010) 39646–39654. doi:10.1074/jbc.M110.164160.
- [93] R.J. Mailloux, W.G. Willmore, S-glutathionylation reactions in mitochondrial function and disease., *Front. Cell Dev. Biol.* 2 (2014) 68. doi:10.3389/fcell.2014.00068.
- [94] E.R. Taylor, F. Hurrell, R.J. Shannon, T.K. Lin, J. Hirst, M.P. Murphy, Reversible glutathionylation of complex I increases mitochondrial superoxide formation, *J. Biol. Chem.* 278 (2003) 19603–19610. doi:10.1074/jbc.M209359200.
- [95] L. Bräutigam, L.D.E. Jensen, G. Poschmann, S. Nyström, S. Bannenberg, K. Dreij, K. Lepka, T. Prozorovski, S.J. Montano, O. Aktas, P. Uhlén, K. Stühler, Y. Cao, A. Holmgren, C. Berndt, Glutaredoxin regulates vascular development by reversible glutathionylation of sirtuin 1., *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 20057–62. doi:10.1073/pnas.1313753110.
- [96] P.T. Kang, L. Zhang, C.L. Chen, J. Chen, K.B. Green, Y.R. Chen, Protein thiyl radical mediates S-glutathionylation of complex i, *Free Radic. Biol. Med.* 53 (2012) 962–973. doi:10.1016/j.freeradbiomed.2012.05.025.
- [97] S.I. Hashemy, C. Johansson, C. Berndt, C.H. Lillig, A. Holmgren, Oxidation and S-nitrosylation of cysteines in human cytosolic and mitochondrial glutaredoxins: Effects on structure and activity, *J. Biol. Chem.* 282 (2007) 14428–14436. doi:10.1074/jbc.M700927200.
- [98] T.R. Hurd, N.J. Costa, C.C. Dahm, S.M. Beer, S.E. Brown, A. Filipovska, M.P. Murphy, Glutathionylation of mitochondrial proteins, *Antioxid. Redox Signal.* 7 (2005) 999–1010. doi:10.1089/ars.2005.7.999.
- [99] T.R. Hurd, R. Requejo, A. Filipovska, S. Brown, T.A. Prime, A.J. Robinson, I.M. Fearnley, M.P. Murphy, Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on Cys-531 and Cys-704 of the 75-kDa subunit: Potential role of Cys residues in decreasing oxidative damage, *J. Biol. Chem.* 283 (2008) 24801–24815. doi:10.1074/jbc.M803432200.
- [100] C. Jacob, I. Knight, P.G. Winyard, Aspects of the biological redox chemistry of cysteine: From simple redox responses to sophisticated signalling pathways, in: *Biol. Chem.*, 2006: pp. 1385–1397.

- doi:10.1515/BC.2006.174.
- [101] N.H.P. Cnubben, I.M.C.M. Rietjens, H. Wortelboer, J. Van Zanden, P.J. Van Bladeren, The interplay of glutathione-related processes in antioxidant defense, *Environ. Toxicol. Pharmacol.* 10 (2001) 141–152. doi:10.1016/S1382-6689(01)00077-1.
- [102] D.P. Jones, Extracellular redox state: refining the definition of oxidative stress in aging, *Rejuvenation Res.* 9 (2006) 169–181. doi:10.1089/rej.2006.9.169.
- [103] D.-F. Dai, Y.A. Chiao, D.J. Marcinek, H.H. Szeto, P.S. Rabinovitch, Mitochondrial oxidative stress in aging and healthspan, *Longev. Heal.* 3 (2014) 6. doi:10.1186/2046-2395-3-6.
- [104] K.C. Kregel, H.J. Zhang, An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292 (2007) R18-36. doi:10.1152/ajpregu.00327.2006.
- [105] V.B.C. Junqueira, S.B.M. Barros, S.S. Chan, L. Rodrigues, L. Giavarotti, R.L. Abud, G.P. Deucher, Aging and oxidative stress, *Mol. Aspects Med.* 25 (2004) 5–16. doi:10.1016/j.mam.2004.02.003.
- [106] M. El Assar, J. Angulo, L. Rodríguez-Mañás, Oxidative stress and vascular inflammation in aging, *Free Radic. Biol. Med.* 65 (2013) 380–401. doi:10.1016/j.freeradbiomed.2013.07.003.
- [107] K.S. SantaCruz, E. Yazlovitskaya, J. Collins, J. Johnson, C. DeCarli, Regional NAD(P)H:quinone oxidoreductase activity in Alzheimer's disease, *Neurobiol. Aging.* 25 (2004) 63–69. doi:10.1016/S0197-4580(03)00117-9.
- [108] D.S. Albers, M.F. Beal, Mitochondrial dysfunction and oxidative stress in aging and neurodegenerative disease, *J. Neural Transm. Suppl.* 59 (2000) 133–154. doi:10.1007/978-3-7091-6781-6_16.
- [109] A.B. Salmon, A. Richardson, V.I. Pérez, Update on the oxidative stress theory of aging: Does oxidative stress play a role in aging or healthy aging?, *Free Radic. Biol. Med.* 48 (2010) 642–655. doi:10.1016/j.freeradbiomed.2009.12.015.
- [110] F.L. Muller, M.S. Lustgarten, Y. Jang, A. Richardson, H. Van Remmen, Trends in oxidative aging theories, *Free Radic. Biol. Med.* 43 (2007) 477–503. doi:10.1016/j.freeradbiomed.2007.03.034.
- [111] S. Herbette, P. Roeckel-Drevet, J.R. Drevet, Seleno-independent glutathione peroxidases: More than simple antioxidant scavengers, *FEBS J.* 274 (2007) 2163–2180. doi:10.1111/j.1742-4658.2007.05774.x.
- [112] R. Brigelius-Flohe, Glutathione peroxidases and redox-regulated transcription factors, in: *Biol. Chem.*, 2006: pp. 1329–1335. doi:10.1515/BC.2006.166.
- [113] X.G. Lei, W.-H. Cheng, J.P. McClung, Metabolic regulation and function of glutathione peroxidase-1, *Annu. Rev. Nutr.* 27 (2007) 41–61. doi:10.1146/annurev.nutr.27.061406.093716.

- [114] B. Halliwell, Antioxidant defence mechanisms: From the beginning to the end (of the beginning), *Free Radic. Res.* 31 (1999) 261–272. doi:10.1080/10715769900300841.
- [115] L.P. Zhang, M. Maiorino, A. Roveri, F. Ursini, Phospholipid Hydroperoxide Glutathione-Peroxidase - Specific Activity in Tissues of Rats of Different Age and Comparison with Other Glutathione Peroxidases, *Biochim. Biophys. Acta.* 1006 (1989) 140–143.
- [116] H. Imai, Y. Nakagawa, Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells, *Free Radic. Biol. Med.* 34 (2003) 145–169. doi:10.1016/S0891-5849(02)01197-8.
- [117] J.Y. Cao, S.J. Dixon, Mechanisms of ferroptosis, *Cell. Mol. Life Sci.* (2016) 1–15. doi:10.1007/s00018-016-2194-1.
- [118] I. Heirman, D. Ginneberge, R. Brigelius-Flohé, N. Hendrickx, P. Agostinis, P. Brouckaert, P. Rottiers, J. Grooten, Blocking tumor cell eicosanoid synthesis by GPx4 impedes tumor growth and malignancy, *Free Radic. Biol. Med.* 40 (2006) 285–294. doi:10.1016/j.freeradbiomed.2005.08.033.
- [119] M.J. Zamek-Gliszczynski, K.A. Hoffmaster, K.I. Nezasa, M.N. Tallman, K.L.R. Brouwer, Integration of hepatic drug transporters and phase II metabolizing enzymes: Mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites, *Eur. J. Pharm. Sci.* 27 (2006) 447–486. doi:10.1016/j.ejps.2005.12.007.
- [120] S. V. Shenvi, B.M. Dixon, K.P. Shay, T.M. Hagen, A rat primary hepatocyte culture model for aging studies, *Curr. Protoc. Toxicol.* (2008). doi:10.1002/0471140856.tx1407s37.
- [121] T. Nguyen, P.J. Sherratt, C.B. Pickett, Regulatory Mechanisms Controlling Gene Expression Mediated By the Antioxidant Response Element, *Annu. Rev. Pharmacol. Toxicol.* 43 (2003) 233–260. doi:10.1146/annurev.pharmtox.43.100901.140229.
- [122] H. Zhang, H.J. Forman, J. Choi, γ -glutamyl transpeptidase in glutathione biosynthesis, *Methods Enzymol.* 401 (2005) 468–483. doi:10.1016/S0076-6879(05)01028-1.
- [123] S. Yadav, E. Zajac, S.S. Singhal, S. Awasthi, Linking stress-signaling, glutathione metabolism, signaling pathways and xenobiotic transporters, *Cancer Metastasis Rev.* 26 (2007) 59–69. doi:10.1007/s10555-007-9043-5.
- [124] J.D. Hayes, J.U. Flanagan, I.R. Jowsey, GLUTATHIONE TRANSFERASES, *Annu. Rev. Pharmacol. Toxicol.* 45 (2005) 51–88. doi:10.1146/annurev.pharmtox.45.120403.095857.
- [125] K.D. Tew, Redox in redux: Emergent roles for glutathione S-transferase P (GSTP) in regulation of cell signaling and S-glutathionylation, *Biochem. Pharmacol.* 73 (2007) 1257–1269. doi:10.1016/j.bcp.2006.09.027.
- [126] P.G. Board, D. Menon, Glutathione transferases, regulators of cellular metabolism and physiology, *Biochim. Biophys. Acta - Gen. Subj.* 1830

- (2013) 3267–3288. doi:10.1016/j.bbagen.2012.11.019.
- [127] Y. Zhu, P.M. Carvey, Z. Ling, Age-related changes in glutathione and glutathione-related enzymes in rat brain, *Brain Res.* 1090 (2006) 35–44. doi:10.1016/j.brainres.2006.03.063.
- [128] N.O. Thomas, K.P. Shay, A.R. Kelley, J.A. Butler, T.M. Hagen, Glutathione maintenance mitigates age-related susceptibility to redox cycling agents, *Redox Biol.* 10 (2016) 45–52. doi:10.1016/j.redox.2016.09.010.
- [129] J.H. Kim, Chemotherapy for colorectal cancer in the elderly, *World J. Gastroenterol.* 21 (2015) 5158–5166. doi:10.3748/wjg.v21.i17.5158.
- [130] D. Ghosh, K.R. Levault, G.J. Brewer, Relative importance of redox buffers GSH and NAD(P)H in age-related neurodegeneration and Alzheimer disease-like mouse neurons, *Aging Cell.* 13 (2014) 631–640. doi:10.1111/accel.12216.
- [131] C. Loguercio, D. Taranto, L.M. Vitale, F. Beneduce, C. Del Vecchio Blanco, Effect of liver cirrhosis and age on the glutathione concentration in the plasma, erythrocytes, and gastric mucosa of man, *Free Radic. Biol. Med.* 20 (1996) 483–488. doi:10.1016/0891-5849(96)02057-6.
- [132] L. Grinberg, E. Fibach, J. Amer, D. Atlas, N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress, *Free Radic. Biol. Med.* 38 (2005) 136–145. doi:10.1016/j.freeradbiomed.2004.09.025.
- [133] S. Hussain, W. Slikker Jr., S.F. Ali, Age-related changes in antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione in different regions of mouse brain, *Int. J. Dev. Neurosci.* 13 (1995) 811–817.
- [134] R. Liu, J. Choi, Age-associated decline in gamma-glutamylcysteine synthetase gene expression in rats., *Free Radic. Biol. Med.* 28 (2000) 566–574. doi:S0891584999002695 [pii].
- [135] S.E. Moriarty-Craige, J. Adkison, M. Lynn, G. Gensler, S. Bressler, D.P. Jones, P. Sternberg, Antioxidant supplements prevent oxidation of cysteine/cystine redox in patients with age-related macular degeneration, *Am. J. Ophthalmol.* 140 (2005) 1020–1026. doi:10.1016/j.ajo.2005.06.043.
- [136] D.P. Jones, V.C. Mody, J.L. Carlson, M.J. Lynn, P. Sternberg, Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses, *Free Radic. Biol. Med.* 33 (2002) 1290–1300. doi:10.1016/S0891-5849(02)01040-7.
- [137] S. V Shenvi, E.J. Smith, T.M. Hagen, Transcriptional regulation of rat gamma-glutamylcysteine ligase catalytic subunit gene is mediated through a distal antioxidant response element, *Pharmacol Res.* 60 (2009) 229–236. doi:S1043-6618(09)00169-8 [pii]\r10.1016/j.phrs.2009.06.003.
- [138] R.S. Sohal, L. Arnold, W.C. Orr, Effect of age on superoxide dismutase, catalase, glutathione reductase, inorganic peroxides, TBA-reactive material, GSH/GSSG, NADPH/NADP⁺ and NADH/NAD⁺ in *Drosophila*

- melanogaster, *Mech. Ageing Dev.* 56 (1990) 223–235. doi:10.1016/0047-6374(90)90084-S.
- [139] H. Zhang, K.J.A. Davies, H.J. Forman, Oxidative stress response and Nrf2 signaling in aging, *Free Radic. Biol. Med.* 88 (2015) 314–336. doi:10.1016/j.freeradbiomed.2015.05.036.
- [140] E.J. Moon, A. Giaccia, Dual roles of NRF2 in tumor prevention and progression: Possible implications in cancer treatment, *Free Radic. Biol. Med.* 79 (2015) 292–299. doi:10.1016/j.freeradbiomed.2014.11.009.
- [141] H. Nohl, Involvement of free radicals in ageing: A consequence or cause of senescence, *Br. Med. Bull.* 49 (1993) 653–667. doi:10.1093/oxfordjournals.bmb.a072638.
- [142] C. Schöneich, Reactive oxygen species and biological aging: A mechanistic approach, *Exp. Gerontol.* 34 (1999) 19–34. doi:10.1016/S0531-5565(98)00066-7.
- [143] A. Vasilaki, A. Mansouri, H. Van Remmen, J.H. van der Meulen, L. Larkin, A.G. Richardson, A. McArdle, J.A. Faulkner, M.J. Jackson, Free radical generation by skeletal muscle of adult and old mice: Effect of contractile activity, *Aging Cell.* 5 (2006) 109–117. doi:10.1111/j.1474-9726.2006.00198.x.
- [144] R.S. Sohal, W.C. Orr, The redox stress hypothesis of aging, *Free Radic. Biol. Med.* 52 (2012) 539–555. doi:10.1016/j.freeradbiomed.2011.10.445.
- [145] J.W. Kaspar, S.K. Niture, A.K. Jaiswal, Nrf2:INrf2 (Keap1) signaling in oxidative stress, *Free Radic. Biol. Med.* 47 (2009) 1304–1309. doi:10.1016/j.freeradbiomed.2009.07.035.
- [146] S.E. Purdum-Dickinson, E.V. Sheveleva, H. Sun, Q.M. Chen, Translational control of nrf2 protein in activation of antioxidant response by oxidants, *Mol. Pharmacol.* 72 (2007) 1074–1081.
- [147] B. Xu, J. Zhang, J. Strom, S. Lee, Q.M. Chen, Myocardial ischemic reperfusion induces de novo Nrf2 protein translation, *Biochim. Biophys. Acta - Mol. Basis Dis.* 1842 (2014) 1638–1647. doi:10.1016/j.bbadis.2014.06.002.
- [148] W. Li, N. Thakor, E.Y. Xu, Y. Huang, C. Chen, R. Yu, M. Holcik, A.N. Kong, An internal ribosomal entry site mediates redox-sensitive translation of Nrf2, *Nucleic Acids Res.* 38 (2009) 778–788. doi:10.1093/nar/gkp1048.
- [149] N. Li, S. Muthusamy, R. Liang, H. Sarojini, E. Wang, Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1, *Mech. Ageing Dev.* 132 (2011) 75–85. doi:10.1016/j.mad.2010.12.004.
- [150] X. Cheng, C.H. Ku, R.C.M. Siow, Regulation of the Nrf2 antioxidant pathway by microRNAs: New players in micromanaging redox homeostasis, *Free Radic. Biol. Med.* 64 (2013) 4–11. doi:10.1016/j.freeradbiomed.2013.07.025.
- [151] S.V. Shenvi, E.J. Smith, T.M. Hagen, Identification of age-specific Nrf2 binding to a novel antioxidant response element locus in the Gclc promoter: a compensatory means for the loss of glutathione synthetic

- capacity in the aging rat liver, *Aging Cell*. 11 (2012) 297–304.
- [152] J.R. Walker, A.I. Su, D.W. Self, J.B. Hogenesch, H. Lapp, R. Maier, D. Hoyer, G. Bilbe, Applications of a rat multiple tissue gene expression data set, *Genome Res.* (n.d.) 742–749.
- [153] M.-K. Kwak, K. Itoh, M. Yamamoto, T.W. Kensler, Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter., *Mol. Cell. Biol.* 22 (2002) 2883–92. doi:10.1128/MCB.22.9.2883.
- [154] W. Hur, N.S. Gray, Small molecule modulators of antioxidant response pathway, *Curr. Opin. Chem. Biol.* 15 (2011) 162–173. doi:10.1016/j.cbpa.2010.12.009.
- [155] M.S. Dhivya Vadhana, S. Siva Arumugam, M. Carloni, C. Nasuti, R. Gabbianelli, Early life permethrin treatment leads to long-term cardiotoxicity, *Chemosphere*. 93 (2013) 1029–1034. doi:10.1016/j.chemosphere.2013.05.073.
- [156] Y. Zhang, R. Munday, Dithiolethiones for cancer chemoprevention: where do we stand?, *Mol. Cancer Ther.* 7 (2008) 3470–3479. doi:10.1158/1535-7163.MCT-08-0625.
- [157] M.A. Mori, P. Raghavan, T. Thomou, J. Boucher, S. Robida-Stubbs, Y. Macotela, S.J. Russell, J.L. Kirkland, T.K. Blackwell, C.R. Kahn, Role of microRNA processing in adipose tissue in stress defense and longevity, *Cell. Metab.* (2012) 336–347.
- [158] S.R. Dalal, J.H. Kwon, The role of microRNA in inflammatory bowel disease, *Gastroenterol. Hepatol.* 6 (2010) 714–722. doi:10.1016/j.smim.2009.01.001.
- [159] P. Jiang, R. Liu, Y. Zheng, X. Liu, L. Chang, S. Xiong, Y. Chu, MiR-34a inhibits lipopolysaccharide-induced inflammatory response through targeting Notch1 in murine macrophages, *Exp. Cell Res.* 318 (2012) 1175–1184. doi:10.1016/j.yexcr.2012.03.018.
- [160] R.M. O’Connell, D.S. Rao, D. Baltimore, microRNA regulation of inflammatory responses., *Annu. Rev. Immunol.* 30 (2012) 295–312. doi:10.1146/annurev-immunol-020711-075013.
- [161] A. Paraskevi, G. Theodoropoulos, I. Papaconstantinou, G. Mantzaris, N. Nikiteas, M. Gazouli, Circulating MicroRNA in inflammatory bowel disease, *J. Crohn’s Colitis*. 6 (2012) 900–904. doi:10.1016/j.crohns.2012.02.006.
- [162] L. a Finlay, A.J. Michels, J. a Butler, E.J. Smith, J.S. Monette, R.F. Moreau, S.K. Petersen, B. Frei, T.M. Hagen, R- α -lipoic acid does not reverse hepatic inflammation of aging, but lowers lipid anabolism, while accentuating circadian rhythm transcript profiles., *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 302 (2012) R587–97. doi:10.1152/ajpregu.00393.2011.
- [163] A. Salminen, K. Kaarniranta, A. Kauppinen, Inflammaging: Disturbed interplay between autophagy and inflammasomes, *Aging (Albany. NY)*. 4 (2012) 166–175. doi:10.18632/aging.100444.
- [164] D. Betel, A. Koppal, P. Agius, C. Sander, C. Leslie, Comprehensive modeling

- of microRNA targets predicts functional non-conserved and non-canonical sites, *Genome Biol.* 11 (2010) R90. doi:10.1186/gb-2010-11-8-r90.
- [165] M. Yang, Y. Yao, G. Eades, Y. Zhang, Q. Zhou, MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism, *Breast Cancer Res. Treat.* 129 (2011) 983–991. doi:10.1007/s10549-011-1604-1.
- [166] D.F. Dai, P.P. Karunadharma, Y.A. Chiao, N. Basisty, D. Crispin, E.J. Hsieh, T. Chen, H. Gu, D. Djukovic, D. Raftery, R.P. Beyer, M.J. Maccoss, P.S. Rabinovitch, Altered proteome turnover and remodeling by short-term caloric restriction or rapamycin rejuvenate the aging heart, *Aging Cell.* 13 (2014) 529–539. doi:10.1111/accel.12203.
- [167] T.M. Hagen, V. Vinarsky, C.M. Wehr, B.N. Ames, (R)-alpha-lipoic acid reverses the age-associated increase in susceptibility of hepatocytes to tert-butylhydroperoxide both in vitro and in vivo, *Antioxid. Redox Signal.* (2000) 473–483.
- [168] C. Geismann, A. Arlt, S. Sebens, H. Schaffer, Cytoprotection “gone astray”: Nrf2 and its role in cancer, *Onco. Targets. Ther.* 7 (2014) 1497–1518. doi:10.2147/OTT.S36624.
- [169] G.S. Wiberg, H.L. Trenholm, B.B. Coldwell, Increased ethanol toxicity in old rats: Changes in LD50, in vivo and in vitro metabolism, and liver alcohol dehydrogenase activity, *Toxicol. Appl. Pharmacol.* 16 (1970) 718–727. doi:10.1016/0041-008X(70)90077-3.
- [170] J.P. Richie, C.A. Lang, T.S. Chen, Acetaminophen-induced depletion of glutathione and cysteine in the aging mouse kidney, *Biochem. Pharmacol.* (1992) 129–135.
- [171] R. Testa, M. Ghia, F. Mattioli, S. Borzone, S. Cagliaris, E. Mereto, E. Giannini, D. Risso, Effects of reduced glutathione and n-acetylcysteine on lidocaine metabolism in cimetidine treated rats., *Fundam. Clin. Pharmacol.* 12 (1998) 220–4. <http://www.ncbi.nlm.nih.gov/pubmed/9565778>.
- [172] J.M. Butler, E.J. Begg, Free drug metabolic clearance in elderly people, *Clin. Pharmacokinet.* 47 (2008) 297–321. doi:10.2165/00003088-200847050-00002.
- [173] A.J. McLachlan, S. Bath, V. Naganathan, S.N. Hilmer, D.G. Le Couteur, S.J. Gibson, F.M. Blyth, Clinical pharmacology of analgesic medicines in older people: Impact of frailty and cognitive impairment, *Br. J. Clin. Pharmacol.* 71 (2011) 351–364. doi:10.1111/j.1365-2125.2010.03847.x.
- [174] N. Li, A.E. Nel, Role of the Nrf2-Mediated Signaling Pathway as a Negative Regulator of Inflammation: Implications for the Impact of Particulate Pollutants on Asthma, *Antioxid. Redox Signal.* 8 (2006) 88–98. doi:10.1089/ars.2006.8.88.
- [175] G.J. Brewer, Epigenetic oxidative redox shift (EORS) theory of aging unifies the free radical and insulin signaling theories, *Exp. Gerontol.* 45 (2010) 173–179. doi:10.1016/j.exger.2009.11.007.
- [176] J. Paredes, D. Jones, M. Wilson, J. Herndon, Age-related alterations of plasma glutathione and oxidation of redox potentials in chimpanzee (*Pan*

- troglodytes) and rhesus monkey (*Macaca mulatta*), *Age*. 36 (2014) 719–732.
- [177] S.J. Stohs, W.A. Al-Turk, C.R. Angle, R.J. Heinicke, Glutathione S-transferase activity in liver, lung and intestinal mucosa of aging female mice, *Gen Pharmacol.* 13 (1982) 519–522. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7152232.
- [178] M. Spearman, K. Leibman, Aging selectively alters glutathione S-transferase isozyme concentrations in liver and lung cytosol, *Drug Metab. Dispos.* 12 (1984) 661–71.
- [179] M.C. Carrillo, M. Nokubo, K. Kitani, K. Satoh, K. Sato, Age-related alterations of enzyme activities and subunits of hepatic glutathione S-transferases in male and female Fischer-344 rats, *Biochim. Biophys. Acta (BBA)/Protein Struct. Mol.* 1077 (1991) 325–331. doi:10.1016/0167-4838(91)90547-D.
- [180] M. Cornet, K. Mertens, A. Callaerts, W. Sonck, A. Vercruysse, V. Rogiers, Age- and gender-related changes in the hepatic metabolism of 2-methylpropene and relationship to epoxide metabolizing enzymes, *Mech. Ageing Dev.* 74 (1994) 103–115. doi:10.1016/0047-6374(94)90102-3.
- [181] S. Patriarca, A.L. Furfaro, L. Cosso, E. Pesce Maineri, E. Balbis, C. Domenicotti, M. Nitti, D. Cottalasso, U.M. Marinari, M.A. Pronzato, N. Traverso, Heme oxygenase 1 expression in rat liver during ageing and ethanol intoxication, *Biogerontology.* 8 (2007) 365–372. doi:10.1007/s10522-006-9079-x.
- [182] E. Ho, K. Karimi Galoughi, C.-C. Liu, R. Bhindi, G. a Figtree, Biological markers of oxidative stress: Applications to cardiovascular research and practice., *Redox Biol.* 1 (2013) 483–91. doi:10.1016/j.redox.2013.07.006.
- [183] J. Liu, W. Qu, M.B. Kadiiska, Role of oxidative stress in cadmium toxicity and carcinogenesis, *Toxicol. Appl. Pharmacol.* 238 (2009) 209–214. doi:10.1016/j.taap.2009.01.029.
- [184] R. V. Sekhar, S. V. McKay, S.G. Patel, A.P. Guthikonda, V.T. Reddy, A. Balasubramanyam, F. Jahoor, Glutathione synthesis is diminished in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine, *Diabetes Care.* 34 (2011) 162–167. doi:10.2337/dc10-1006.
- [185] D. Ghosh, K.R. LeVault, A.J. Barnett, G.J. Brewer, A reversible early oxidized redox state that precedes macromolecular ROS damage in aging nontransgenic and 3xTg-AD mouse neurons., *J. Neurosci.* 32 (2012) 5821–32. doi:10.1523/JNEUROSCI.6192-11.2012.
- [186] J. Yoshino, K.F. Mills, M.J. Yoon, S.I. Imai, Nicotinamide mononucleotide, a key NAD + intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice, *Cell Metab.* 14 (2011) 528–536. doi:10.1016/j.cmet.2011.08.014.
- [187] C. Lind, P. Hochstein, L. Ernster, DT-diaphorase as a quinone reductase: A cellular control device against semiquinone and superoxide radical

- formation, *Arch. Biochem. Biophys.* 216 (1982) 178–185. doi:10.1016/0003-9861(82)90202-8.
- [188] H. Thor, M.T. Smith, P. Hartzell, G. Bellomo, S.A. Jewell, S. Orrenius, The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells, *J. Biol. Chem.* 257 (1982) 12419–12425.
- [189] D.S. Kalinowski, C. Stefani, S. Toyokuni, T. Ganz, G.J. Anderson, N. V. Subramaniam, D. Trinder, J.K. Olynyk, A. Chua, P.J. Jansson, S. Sahni, D.J.R. Lane, A.M. Merlot, Z. Kovacevic, M.L.H. Huang, C.S. Lee, D.R. Richardson, Redox cycling metals: Pedaling their roles in metabolism and their use in the development of novel therapeutics, *Biochim. Biophys. Acta - Mol. Cell Res.* 1863 (2016) 727–748. doi:10.1016/j.bbamcr.2016.01.026.
- [190] K. Jomova, M. Valko, Advances in metal-induced oxidative stress and human disease, *Toxicology.* 283 (2011) 65–87. doi:10.1016/j.tox.2011.03.001.
- [191] N. Ercal, H. Gurer-Orhan, N. Aykin-Burns, Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage, *Curr. Top. Med. Chem.* 1 (2001) 529–539. doi:http://dx.doi.org/10.2174/1568026013394831.
- [192] D. Bonne-Barkay, W.J. Langston, D.A. Di Monte, Toxicity of redox cycling pesticides in primary mesencephalic cultures, *Antioxid.Redox.Signal.* 7 (2005) 649–653. doi:10.1089/ars.2005.7.649.
- [193] M.E. DeLorenzo, G.I. Scott, P.E. Ross, Toxicity of pesticides to aquatic microorganisms: a review., *Environ. Toxicol. Chem.* 20 (2001) 84–98. doi:10.1002/etc.5620200108.
- [194] P. Kovacic, R. Somanathan, Mechanism of Anesthetic Toxicity: Metabolism, Reactive Oxygen Species, Oxidative Stress, and Electron Transfer, *ISRN Anesthesiol.* 2011 (2011).
- [195] T. Michalska, I. Kruk, K. Lichszteid, J. Kladny, Hydroxyl radical generation by redox cycling of some chemotherapeutic antibiotics, *Toxicol. Environ. Chem.* 56 (1996) 171–176. <http://www.scopus.com/inward/record.url?eid=2-s2.0-0030483429&partnerID=40&md5=8c32caa5d8abab42d1a6736b2285fb4e>.
- [196] M. Sack, L. Alili, E. Karaman, S. Das, A. Gupta, S. Seal, P. Brenneisen, Combination of conventional chemotherapeutics with redox-active cerium oxide nanoparticles--a novel aspect in cancer therapy., *Mol. Cancer Ther.* 13 (2014) 1740–9. doi:10.1158/1535-7163.MCT-13-0950.
- [197] C. von Montfort, L. Alili, S. Teuber-Hanselmann, P. Brenneisen, Redox-active cerium oxide nanoparticles protect human dermal fibroblasts from PQ-induced damage, *Redox Biol.* 4 (2015) 1–5. doi:10.1016/j.redox.2014.11.007.
- [198] G.T. Wondrak, Redox-directed cancer therapeutics: molecular mechanisms

- and opportunities., *Antioxid. Redox Signal.* 11 (2009) 3013–69. doi:10.1089/ars.2009.2541.
- [199] H. Zubair, H.Y. Khan, a Sohail, S. Azim, M.F. Ullah, a Ahmad, F.H. Sarkar, S.M. Hadi, Redox cycling of endogenous copper by thymoquinone leads to ROS-mediated DNA breakage and consequent cell death: putative anticancer mechanism of antioxidants., *Cell Death Dis.* 4 (2013) e660. doi:10.1038/cddis.2013.172.
- [200] H. Wildiers, Mastering chemotherapy dose reduction in elderly cancer patients, *Eur. J. Cancer.* 43 (2007) 2235–2241. doi:10.1016/j.ejca.2007.06.013.
- [201] U. Wedding, F. Honecker, C. Bokemeyer, L. Pientka, K. Höffken, Tolerance to chemotherapy in elderly patients with cancer, *Cancer Control.* 14 (2007) 44–56.
- [202] C.-H. Köhne, G. Folprecht, R.M. Goldberg, E. Mitry, P. Rougier, Chemotherapy in elderly patients with colorectal cancer., *Oncologist.* 13 (2008) 390–402. doi:10.1634/theoncologist.2007-0043.
- [203] R. Meza, J. Jeon, S.H. Moolgavkar, E.G. Luebeck, Age-specific incidence of cancer: Phases, transitions, and biological implications., *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 16284–16289. doi:10.1073/pnas.0801151105.
- [204] D. Siegel, J.K. Kepa, D. Ross, Biochemical and genetic analysis of NAD(P)H:quinone oxidoreductase 1 (NQO1)., *Curr. Protoc. Toxicol.* Chapter 4 (2007) Unit4.22. doi:10.1002/0471140856.tx0422s32.
- [205] S.H.Y. Wong, J.A. Knight, S.M. Hopfer, O. Zaharia, C.N. Leach, F.W. Sunderman, Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde-thiobarbituric acid adduct, *Clin. Chem.* 33 (1987) 214–220.
- [206] O. Sommerburg, T. Grune, S. Klee, F.R. Ungemach, W.G. Siems, Formation of 4-hydroxynonenal and further aldehydic mediators of inflammation during bromotrichloromethane treatment of rat liver cells, *Mediators Inflamm.* 2 (1993) 27–31. doi:10.1155/S0962935193000031.
- [207] M.W. Fariss, D.J. Reed, High-performance liquid chromatography of thiols and disulfides: Dinitrophenol derivatives, *Methods Enzymol.* 143 (1987) 101–109. doi:10.1016/0076-6879(87)43018-8.
- [208] H. Zhu, K. Itoh, M. Yamamoto, J.L. Zweier, Y. Li, Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: Protection against reactive oxygen and nitrogen species-induced cell injury, *FEBS Lett.* 579 (2005) 3029–3036. doi:10.1016/j.febslet.2005.04.058.
- [209] V. V Vnukov, O.I. Gutsenko, N.P. Milutina, A.A. Ananyan, A.O. Danilenko, S.B. Panina, I. V Kornienko, Influence of SkQ1 on Expression of Nrf2 Transcription Factor Gene, ARE-Controlled Genes of Antioxidant Enzymes and Their Activity in Rat Blood Leukocytes., *Biochem.* 80 (2015) 586–91. doi:10.1134/S0006297915050107.
- [210] J.Y. Chan, M. Kwong, Impaired expression of glutathione synthetic enzyme

- genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein, *Biochim. Biophys. Acta - Gene Struct. Expr.* 1517 (2000) 19–26. doi:10.1016/S0167-4781(00)00238-4.
- [211] M.H. Kweon, Y. In Park, H.C. Sung, H. Mukhtar, The novel antioxidant 3-O-caffeoyl-1-methylquinic acid induces Nrf2-dependent phase II detoxifying genes and alters intracellular glutathione redox, *Free Radic. Biol. Med.* 40 (2006) 1349–1361. doi:10.1016/j.freeradbiomed.2005.12.002.
- [212] D. Glass, A. Viñuela, M.N. Davies, A. Ramasamy, L. Parts, D. Knowles, A.A. Brown, A.K. Hedman, K.S. Small, A. Buil, E. Grundberg, A.C. Nica, P. Di Meglio, F.O. Nestle, M. Ryten, R. Durbin, M.I. McCarthy, P. Deloukas, E.T. Dermitzakis, M.E. Weale, V. Bataille, T.D. Spector, Gene expression changes with age in skin, adipose tissue, blood and brain., *Genome Biol.* 14 (2013) R75. doi:10.1186/gb-2013-14-7-r75.
- [213] B. Halliwell, J.M.C. Gutteridge, *Free Radicals in Biology and Medicine*, 2007. doi:10.1016/0891-5849(91)90055-8.
- [214] J. Leakey, H. Cunny, J. Bazare Jr, P. Webb, J. Lipscomb, W. Slikker Jr, R. Feuers, P. Duffy, R. Hart, Effects of aging and caloric restriction on hepatic drug metabolizing enzymes in the fischer 344 rat II: Effects on conjugating enzymes, *Mech. Ageing Dev.* 48 (1989) 157–166.
- [215] F. Amicarelli, C. Di Ilio, L. Masciocco, A. Bonfigli, O. Zarivi, M.R. D’Andrea, C. Di Giulio, M. Miranda, Aging and detoxifying enzymes responses to hypoxic or hyperoxic treatment, *Mech. Ageing Dev.* 97 (1997) 215–226. doi:10.1016/S0047-6374(97)00061-4.
- [216] E. Vyskocilova, B. Szotakova, L. Skalova, H. Bartikova, J. Hlavacova, I. Bousova, Age-related changes in hepatic activity and expression of detoxification enzymes in male rats, *Biomed Res. Int.* 2013 (2013). doi:10.1155/2013/408573.
- [217] E. Cadenas, Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism, *Biochem. Pharmacol.* 49 (1995) 127–140. doi:10.1016/S0006-2952(94)00333-5.
- [218] H. Morrison, B. Jernstrom, M. Nordenskjold, H. Thor, S. Orrenius, Induction of dna damage by menadione (2-methyl-1,4-naphthoquinone) in primary cultures of rat hepatocytes, *Biochem. Pharmacol.* 33 (1984) 1763–1769. doi:10.1016/0006-2952(84)90347-2.
- [219] H. Morrison, D. DI Monte, M. Nordenskjold, B. Jernstrom, Induction of cell damage by menadione and benzo(a)-pyrene-3,6-quinone in cultures of adult rat hepatocytes and human fibroblasts, *Toxicol. Lett.* 28 (1985) 37–47. doi:10.1016/0378-4274(85)90007-4.
- [220] B. Frei, K.H. Winterhalter, C. Richter, Menadione- (2-methyl-1,4-naphthoquinone-) dependent enzymatic redox cycling and calcium release by mitochondria., *Biochemistry.* 25 (1986) 4438–43. <http://www.ncbi.nlm.nih.gov/pubmed/3092856>.
- [221] P.F. Smith, D.W. Alberts, G.F. Rush, Menadione-induced oxidative stress in hepatocytes isolated from fed and fasted rats: The role of NADPH-

- regenerating pathways, *Toxicol. Appl. Pharmacol.* 89 (1987) 190–201. doi:10.1016/0041-008X(87)90040-8.
- [222] H. Wefers, H. Sies, Hepatic low-level chemiluminescence during redox cycling of menadione and the menadione-glutathione conjugate: Relation to glutathione and NAD(P)H:quinone reductase (DT-diaphorase) activity, *Arch. Biochem. Biophys.* 224 (1983) 568–578. doi:10.1016/0003-9861(83)90244-8.
- [223] D. Ross, H. Thor, S. Orrenius, P. Moldeus, Interaction of menadione (2-methyl-1,4-naphthoquinone) with glutathione, *Chem. Biol. Interact.* 55 (1985) 177–184. doi:10.1016/S0009-2797(85)80126-5.
- [224] J.H. Chung, D.C. Seo, S.H. Chung, J.Y. Lee, S. a Seung, Metabolism and cytotoxicity of menadione and its metabolite in rat platelets., *Toxicol. Appl. Pharmacol.* 142 (1997) 378–85. doi:10.1006/taap.1996.8048.
- [225] T.J. Chiou, W.F. Tzeng, The roles of glutathione and antioxidant enzymes in menadione-induced oxidative stress, *Toxicology.* 154 (2000) 75–84. doi:10.1016/S0300-483X(00)00321-8.
- [226] P.H. Shih, G.C. Yen, Differential expressions of antioxidant status in aging rats: The role of transcriptional factor Nrf2 and MAPK signaling pathway, *Biogerontology.* 8 (2007) 71–80. doi:10.1007/s10522-006-9033-y.
- [227] D.-H. Hyun, S.S. Emerson, D.-G. Jo, M.P. Mattson, R. de Cabo, Calorie restriction up-regulates the plasma membrane redox system in brain cells and suppresses oxidative stress during aging., *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 19908–12. doi:10.1073/pnas.0608008103.
- [228] B.T. Tung, E. Rodriguez-Bies, M. Ballesteros-Simarro, V. Motilva, P. Navas, G. Lopez-Lluch, Modulation of endogenous antioxidant activity by resveratrol and exercise in mouse liver is age dependent, *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* 69 (2014) 398–409. doi:10.1093/gerona/glt102.
- [229] L. Ernster, DT diaphorase, *Methods Enzymol.* 10 (1967) 309–317.
- [230] Y. Li, Regulation of human NAD(P)H:Quinone oxidoreductase gene: Role of api binding site contained within human antioxidant response element, *J. Biol. Chem.* 267 (1992) 15097–15104.
- [231] T. Xie, M. Belinsky, Y. Xu, A.K. Jaiswal, ARE- and TRE-mediated regulation of gene expression: Response to xenobiotics and antioxidants, *J. Biol. Chem.* 270 (1995) 6894–6900.
- [232] M.Y. Farooqui, W.W. Day, D.M. Zamorano, Glutathione and lipid peroxidation in the aging rat., *Comp. Biochem. Physiol. B.* 88 (1987) 177–180.
- [233] R. Liu, Down-regulation of gamma-glutamylcysteine synthetase regulatory subunit gene expression in rat brain tissue during aging., *J. Neurosci. Res.* 68 (2002) 344–351. doi:10.1002/jnr.10217.
- [234] M. Erden-Inal, E. Sunal, G. Kanbak, Age-related changes in the glutathione redox system., *Cell Biochem. Funct.* 20 (2002) 61–6. doi:10.1002/cbf.937.
- [235] J.O. Miners, R. Drew, D.J. Birkett, Mechanism of action of paracetamol

- protective agents in mice in vivo, *Biochem. Pharmacol.* 33 (1984) 2995–3000. doi:10.1016/0006-2952(84)90599-9.
- [236] S. Pratt, C. Ioannides, Mechanism of the protective action of n-acetylcysteine and methionine against paracetamol toxicity in the hamster, *Arch. Toxicol.* 57 (1985) 173–177. doi:10.1007/BF00290883.
- [237] E. Gouix, A. Buisson, A. Nieoullon, L. Kerkerian-Le Goff, J.S. Tauskela, N. Blondeau, L. Had-Aissouni, Oxygen glucose deprivation-induced astrocyte dysfunction provokes neuronal death through oxidative stress, *Pharmacol. Res.* 87 (2014) 8–17. doi:10.1016/j.phrs.2014.06.002.
- [238] P. Costantini, B. V. Chernyak, V. Petronilli, P. Bernardi, Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites, *J. Biol. Chem.* 271 (1996) 6746–6751. doi:10.1074/jbc.271.12.6746.
- [239] A.P. Halestrap, K.Y. Woodfield, C.P. Connern, Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase, *J. Biol. Chem.* 272 (1997) 3346–3354. doi:10.1074/jbc.272.6.3346.
- [240] D.R. Green, J.C. Reed, Mitochondria and apoptosis. [Review] [59 refs], *Science* (80-.). 281 (1998) 1309–1312.
- [241] M.W. Fariss, C.B. Chan, M. Patel, B. Van Houten, S. Orrenius, Role of mitochondria in toxic oxidative stress., *Mol. Interv.* 5 (2005) 94–111. doi:10.1124/mi.5.2.7.
- [242] M. Ott, V. Gogvadze, S. Orrenius, B. Zhivotovsky, Mitochondria, oxidative stress and cell death, *Apoptosis.* 12 (2007) 913–922. doi:10.1007/s10495-007-0756-2.
- [243] A.-L. Nieminen, Apoptosis and necrosis in health and disease: Role of mitochondria, *Int. Rev. Cytol.* 224 (2003) 29–55. doi:10.1016/S0074-7696(05)24002-0.
- [244] J.J. Lemasters, T.P. Theruvath, Z. Zhong, A.-L. Nieminen, Mitochondrial calcium and the permeability transition in cell death., *Biochim. Biophys. Acta.* 1787 (2009) 1395–1401. doi:10.1016/j.bbabi.2009.06.009.
- [245] C. Bémeur, J. Vaquero, P. Desjardins, R.F. Butterworth, N-Acetylcysteine attenuates cerebral complications of non-acetaminophen- induced acute liver failure in mice: Antioxidant and anti-inflammatory mechanisms, *Metab. Brain Dis.* 25 (2010) 241–249. doi:10.1007/s11011-010-9201-2.
- [246] L.J. Chun, M.J. Tong, R.W. Busuttill, J.R. Hiatt, Acetaminophen hepatotoxicity and acute liver failure., *J. Clin. Gastroenterol.* 43 (2009) 342–349. doi:10.1097/MCG.0b013e31818a3854.
- [247] R.J. Fontana, Acute liver failure due to drugs, *Semin. Liver Dis.* 28 (2008) 175–187. doi:10.1055/s-2008-1073117.
- [248] R.H. Swerdlow, J.M. Burns, S.M. Khan, The Alzheimer’s disease mitochondrial cascade hypothesis: Progress and perspectives, *Biochim. Biophys. Acta - Mol. Basis Dis.* 1842 (2014) 1219–1231.

- doi:10.1016/j.bbadis.2013.09.010.
- [249] M.F. Beal, Mitochondria take center stage in aging and neurodegeneration, *Ann. Neurol.* 58 (2005) 495–505. doi:10.1002/ana.20624.
- [250] C. Desler, T.L. Hansen, J.B. Frederiksen, M.L. Marcker, K.K. Singh, L. Juel Rasmussen, Is there a link between mitochondrial reserve respiratory capacity and aging?, *J. Aging Res.* 2012 (2012). doi:10.1155/2012/192503.
- [251] C. García-Ruiz, A. Morales, A. Ballesta, J. Rodés, N. Kaplowitz, J.C. Fernández-Checa, Effect of chronic ethanol feeding on glutathione and functional integrity of mitochondria in periportal and perivenous rat hepatocytes, *J. Clin. Invest.* 94 (1994) 193–201. doi:10.1172/JCI117306.
- [252] J. Sastre, F. V. Pallardó, J. Viña, The role of mitochondrial oxidative stress in aging, *Free Radic. Biol. Med.* 35 (2003) 1–8. doi:10.1016/S0891-5849(03)00184-9.
- [253] B.M. Dixon, S.H. Heath, R. Kim, J.H. Suh, T.M. Hagen, Assessment of endoplasmic reticulum glutathione redox status is confounded by extensive ex vivo oxidation, *Antioxid. Redox Signal.* (2008) 963–972.
- [254] J.H. Suh, S.H. Heath, T.M. Hagen, Two subpopulations of mitochondria in the aging rat heart display heterogenous levels of oxidative stress, *Free Radic. Biol. Med.* 35 (2003) 1064–1072. doi:10.1016/S0891-5849(03)00468-4.
- [255] J.P. Monteiro, A.F. Martins, C. Nunes, C.M. Morais, M. Lúcio, S. Reis, T.J.T. Pinheiro, C.F.G.C. Geraldes, P.J. Oliveira, A.S. Jurado, A biophysical approach to menadione membrane interactions: Relevance for menadione-induced mitochondria dysfunction and related deleterious/therapeutic effects, *Biochim. Biophys. Acta - Biomembr.* 1828 (2013) 1899–1908. doi:10.1016/j.bbamem.2013.04.006.
- [256] B.S. Andersson, D.P. Jones, Use of digitonin fractionation to determine mitochondrial transmembrane ion distribution in cells during anoxia., *Anal. Biochem.* (1985) 164–172.
- [257] T.M. Hagen, D.L. Yowe, J.C. Bartholomew, C.M. Wehr, K.L. Do, J.Y. Park, B.N. Ames, Mitochondrial decay in hepatocytes from old rats: Membrane potential declines, heterogeneity and oxidants increase, *Proc. Natl. Acad. Sci. U. S. A.* 94 (1997) 3064–3069. doi:10.1073/pnas.94.7.3064.
- [258] H. Nagai, K. Matsumaru, G. Feng, N. Kaplowitz, Reduced glutathione depletion causes necrosis and sensitization to tumor necrosis factor- α -induced apoptosis in cultured mouse hepatocytes., *Hepatology.* 36 (2002) 55–64. doi:10.1053/jhep.2002.33995.
- [259] M. Vairetti, A. Ferrigno, R. Bertone, P. Richelmi, F. Bertè, I. Freitas, Apoptosis vs. necrosis: Glutathione-mediated cell death during rewarming of rat hepatocytes, *Biochim. Biophys. Acta - Mol. Basis Dis.* 1740 (2005) 367–374. doi:10.1016/j.bbadis.2004.11.022.
- [260] J.H. Suh, S. V. Shenvi, B.M. Dixon, H. Liu, A.K. Jaiswal, R.-M. Liu, T.M. Hagen, Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid, *Proc. Natl. Acad.*

- Sci. 101 (2004) 3381–3386. doi:10.1073/pnas.0400282101.
- [261] E. V Zubkova, B. Robaire, Effect of glutathione depletion on antioxidant enzymes in the epididymis, seminal vesicles, and liver and on spermatozoa motility in the Aging Brown Norway rat, *Biol. Reprod.* 71 (2004) 1002–1008. doi:10.1095/biolreprod.104.028373.
- [262] M.D. Brand, D.G. Nicholls, Assessing mitochondrial dysfunction in cells., *Biochem. J.* 435 (2011) 297–312. doi:10.1042/BJ20110162.
- [263] D. Siegel, J.K. Kepa, D. Ross, NAD(P)H:Quinone Oxidoreductase 1 (NQO1) Localizes to the Mitotic Spindle in Human Cells, *PLoS One.* 7 (2012). doi:10.1371/journal.pone.0044861.
- [264] K. Nomura, H. Imai, T. Koumura, M. Arai, Y. Nakagawa, Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway, *J. Biol. Chem.* 274 (1999) 29294–29302. doi:10.1074/jbc.274.41.29294.
- [265] M. Arai, H. Imai, T. Koumura, M. Yoshida, K. Emoto, M. Umeda, N. Chiba, Y. Nakagawa, Mitochondrial phospholipid hydroperoxide glutathione peroxidase plays a major role in preventing oxidative injury to cells, *J. Biol. Chem.* 274 (1999) 4924–4933. doi:10.1074/jbc.274.8.4924.
- [266] M. Marí, A. Morales, A. Colell, C. García-Ruiz, N. Kaplowitz, J.C. Fernández-Checa, Mitochondrial glutathione: Features, regulation and role in disease, *Biochim. Biophys. Acta - Gen. Subj.* 1830 (2013) 3317–3328. doi:10.1016/j.bbagen.2012.10.018.
- [267] R.J. Mailloux, E.L. Seifert, F. Bouillaud, C. Aguer, S. Collins, M.E. Harper, Glutathionylation acts as a control switch for uncoupling proteins UCP2 and UCP3, *J. Biol. Chem.* 286 (2011) 21865–21875. doi:10.1074/jbc.M111.240242.
- [268] R.J. Mailloux, J.Y. Xuan, B. Beauchamp, L. Jui, M. Lou, M.E. Harper, Glutaredoxin-2 is required to control proton leak through uncoupling protein-3, *J. Biol. Chem.* 288 (2013) 8365–8379. doi:10.1074/jbc.M112.442905.
- [269] P.T. Kang, C.L. Chen, P. Ren, G. Guarini, Y.R. Chen, BCNU-induced gR2 DEFECT mediates S-glutathionylation of Complex i and respiratory uncoupling in myocardium, *Biochem. Pharmacol.* 89 (2014) 490–502. doi:10.1016/j.bcp.2014.03.012.
- [270] M. Seco-Cervera, M. Spis, J.L. García-Giménez, J.S. Ibañez-Cabellos, A. Velázquez-Ledesma, I. Esmorís, S. Bañuls, G. Pérez-Machado, F. V. Pallardó, Oxidative stress and antioxidant response in fibroblasts from Werner and Atypical Werner Syndromes, *Aging (Albany. NY).* 6 (2014) 231–245. doi:10.18632/aging.100649.
- [271] D.G. Nicholls, Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons, *J. Biol. Chem.* 281 (2006) 14864–14874. doi:10.1074/jbc.M510916200.
- [272] D.G. Nicholls, S.L. Budd, Mitochondria and Neuronal Survival, *Physiol. Rev.*

- 80 (2000) 315–360.
<http://physrev.physiology.org/content/80/1/315.abstract>.
- [273] S.W. Perry, J.P. Norman, J. Barbieri, E.B. Brown, H.A. Gelbard, Mitochondrial membrane potential probes and the proton gradient: A practical usage guide, *Biotechniques*. 50 (2011) 98–115. doi:10.2144/000113610.
- [274] National Center for Health Statistics (U.S.), Health, United States, 2004. Special excerpt: trend tables on 65 and older population., 2004.
- [275] J. Lubitz, L. Cai, E. Kramarow, H. Lentzner, Health, life expectancy, and health care spending among the elderly., *N. Engl. J. Med.* 349 (2003) 1048–1055. doi:10.1056/NEJMsa020614.
- [276] W.S. Yang, K.J. Kim, M.M. Gaschler, M. Patel, M.S. Shchepinov, B.R. Stockwell, Peroxidation of polyunsaturated fatty acids by lipoxygenases drives ferroptosis, *Proc. Natl. Acad. Sci.* 113 (2016) E4966–E4975. doi:10.1073/pnas.1603244113.
- [277] Y. Ito, O. Kajkenova, R.J. Feuers, K.B. Udupa, V.G. Desai, J. Epstein, R.W. Hart, D.A. Lipschitz, Impaired glutathione peroxidase activity accounts for the age-related accumulation of hydrogen peroxide in activated human neutrophils, *J Gerontol A Biol Sci Med Sci.* 53 (1998) M169-75. <http://www.ncbi.nlm.nih.gov/pubmed/9597047>.
- [278] D.R. Scott, L.N. Karageuzian, P.J. Anderson, D.L. Epstein, Glutathione peroxidase of calf trabecular meshwork, *Investig. Ophthalmol. Vis. Sci.* 25 (1984) 599–602.
- [279] R.A. Lawrence, R.F. Burk, Glutathione peroxidase activity in selenium-deficient rat liver, *Biochem. Biophys. Res. Commun.* 71 (1976) 952–958. doi:10.1016/0006-291X(76)90747-6.
- [280] S. Pierce, A.L. Tappel, Glutathione peroxidase activities from rat liver, *Biochim. Biophys. Acta - Enzymol.* 523 (1978) 27–36. doi:10.1016/0005-2744(78)90005-0.