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Abstract approved:

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Tory M. Hagen

Glutathione (GSH) is the predominant low molecular weight thiol antioxidant in liver tissue. GSH plays an important role in maintaining the intracellular thiol redox ratio as well as detoxification of electrophiles and xenobiotics. Aging leads to a significant decline (35%; $P \leq 0.05$) in hepatocellular GSH levels. Using young (2-4 months corresponding to an adolescent human) and old (24-28 months corresponding to a 60-75 year old person) male Fischer 344 rats, we determined that the age-related loss of GSH levels were due to lower activity ($53 \pm 6\%$; $P \leq 0.05$) and levels of the rate-limiting enzyme, γ-glutamate cysteine ligase (GCL). GCL is composed of a catalytic subunit (GCLC) and also a modulatory subunit (GCLM) that affects the K_M of its substrate. Since GCLC levels are regulated by transcription, we sought to elucidate its precise transcriptional mechanism and whether aging alters the transcriptome of the enzyme subunit.

A cis-acting DNA sequence called the antioxidant response element (ARE) has been previously implicated in the transcriptional regulation of Phase II enzymes, including GCLC and GCLM. Computer-based analysis of the promoter region of *Gclc* revealed the presence of three putative AREs and a single cis element (ARE-like) containing the core but not the flanking nucleotides of the ARE. Results from experiments where H4IIE rat hepatoma cells were transfected with luciferase reporter constructs containing individual *Gclc* ARE elements revealed that only the ARE element 3.9 kb upstream of the transcriptional start site (ARE3) possessed basal transcriptional activity. Electromobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) experiments on liver tissue and primary hepatocytes in culture showed that NF-E2-related factor 2 (Nrf2) was the predominant transcription factor bound to ARE3 and was partnered with small maf proteins, c-Jun, c-Fos and the histone acetyltransferase CREB-Binding Protein (CBP).

In aging, nuclear steady-state levels of Nrf2 showed a profound 51 $\pm$ 7% ($P \leq 0.0001$) decline leading to lower Nrf2-ARE3 binding (40%) and transcriptional activity (70 $\pm$ 10%; $P \leq 0.05$), consistent with the loss in GCLC levels. Concomitantly, the transcriptional repressor Bach1 was enriched at the ARE3 site and was accompanied by a loss of CBP. These results show that a negative remodeling of the active *Gclc* transcriptional complex occurs in the liver of old rats. Furthermore, Nrf2 was detected at the ARE-like site, which was not transcriptionally active in hepatocytes from young rats. Thus, a promoter switching mechanism may occur with age.

In previously published reports, we demonstrated that administration of the dithiol compound *R*-alpha-lipoic acid (LA; 40 mg/kg body weight; intraperitoneal injection) to old rats reversed the age-related decline in hepatic glutathione levels. LA admininstration both to old rats and to hepatocytes in primary cell culture (100 μM) replenished nuclear Nrf2 levels lost during aging. Additionally, LA increased Nrf2 enrichment and activity of both the ARE3 and ARE-like promoters albeit, to a greater extent at the ARE-like promoter (60 $\pm$ 10%; $P \leq 0.05$). This was accompanied by reversal of the age-related decline in GCLC expression, protein levels and GCL activity. Thus, LA maintains hepatic GSH status during aging by permitting normal ARE-mediated GCLC expression, suggesting that it would be a good therapeutic agent to restore GSH-dependent detoxification systems during aging.

Age-Related Alterations in Transcriptional Regulation of Hepatic Glutathione Synthesis: Remediation by *R*-Alpha-Lipoic Acid

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APPROVED:

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Chair of the Molecular and Cellular Biology Program

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Swapna V. Shenvi, Author

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Chapter 1

General Introduction

1.1 **Background and significance**

The 'free radical theory' of aging

Aging is a multi-factorial process characterized by a progressively reduced ability to cope with physiological challenges (1-6). This leads to a pronounced increase in incidence of most conditions causing morbidity and mortality. The 'Free Radical Theory' of aging proposed by Harman in 1956 (7, 8) suggests that endogenous reactive oxygen species (ROS), which primarily result from normal mitochondrial metabolism, cause progressive damage resulting in the functional decline that defines aging. Accordingly, there are numerous studies that show an increase in oxidative stress and the accumulation of oxidatively modified and dysfunctional macromolecules in older cells (9-11). For example, measurements of oxidant generation in isolated rat hepatocytes from young and old animals employing an intracellular dye that fluoresces upon oxidation, have confirmed that cellular oxidant generation seems to increase with age (12). Because macromolecules that are targeted by free radicals are important in metabolism, cell signaling, and transcriptional control (13-16), their irreversible oxidation is likely to play a critical role in the aging process and in the progression of degenerative diseases associated with aging (17-19).

ROS encompass a variety of diverse chemical species including superoxide anions, hydroxyl radicals and hydrogen peroxide (9, 15, 20, 21). The burden of ROS production is largely counteracted by an intricate antioxidant defense system (9, 21).

Examples of well-characterized antioxidant enzymes include superoxide dismutase (SOD), catalase and glutathione peroxidase (22-31). SOD speeds the conversion of superoxide to hydrogen peroxide, whereas catalase and glutathione peroxidase convert hydrogen peroxide to water (27, 32, 33). A variety of other non-enzymatic, low molecular mass molecules are important in scavenging ROS. These include ascorbate, pyruvate, flavonoids, carotenoids and perhaps most importantly, glutathione (GSH), which is present in millimolar concentrations within cells (34, 35). The balance between ROS production and antioxidant defenses determines the degree of oxidative stress and the extent of modification to cellular proteins, lipids, and DNA (36).

Thus, a ubiquitous antioxidant defense against oxidative damage is central to maintenance of life. This universality of antioxidant defenses strongly supports the free radical theory of aging. The combination of antioxidant systems deployed differs not only between organisms and tissues, but also between cellular compartments. The fact that antioxidant defenses are not uniform further supports the free radical theory of aging; differences in antioxidant systems explain disparities between species' lifespan (4, 37-39). Equally notable is the observation that antioxidant defenses fail in the elderly and inadequately respond to oxidative and toxicological insults, elevating risk for both acute and chronic diseases. For example, there is growing evidence that many chemotherapeutic agents and drugs are less well tolerated in elderly patients than by younger individuals (40, 41). This reduced stress tolerance in the elderly could result from a blunted ability to synthesize protective intracellular antioxidants and detoxification enzymes (42).

Based on the above information, one of the most intuitive approaches to restore stress response in the elderly would be to replenish cellular antioxidant and toxicological defenses that are lost with age. In the case of antioxidant enzymes and certain stress response genes, lifespan of *C. elegans*, *Drosophila* and rodents were extended when either antioxidant enzymes (catalase and superoxide dismutase) were over-expressed or when certain mutations (daf, $p66^{shc}$) were introduced that actually increased cell resistance to oxidative insult (43-46). But, antioxidant defense systems and cellular repair systems have shown to be induced in response to oxidative challenges (47, 48). In this regard, the intracellular generation of oxidants capable of limiting stress response during aging appears paradoxical, especially due to the highly inducible nature of antioxidant systems. Nowhere is this aspect more striking than the decline of the low molecular weight antioxidant GSH and GSH-dependent detoxification systems with age.

Age-related decline in GSH levels

At concentrations between 0.5 and 10 mM, the tripeptide GSH (γ-L-glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol found ubiquitously in tissues of higher organisms and helps to maintain intracellular thiol redox balance (14, 49-54) (Figure 1.1). GSH plays multiple roles in the protection of cells from ROS. These can be listed as 1) detoxification of electrophiles and ROS, 2) maintenance of thiol redox balance during normal homeostasis as well as oxidative stress, 3) scavenging free radicals, and 4) providing an intracellular cysteine reservoir (52, 55,56).

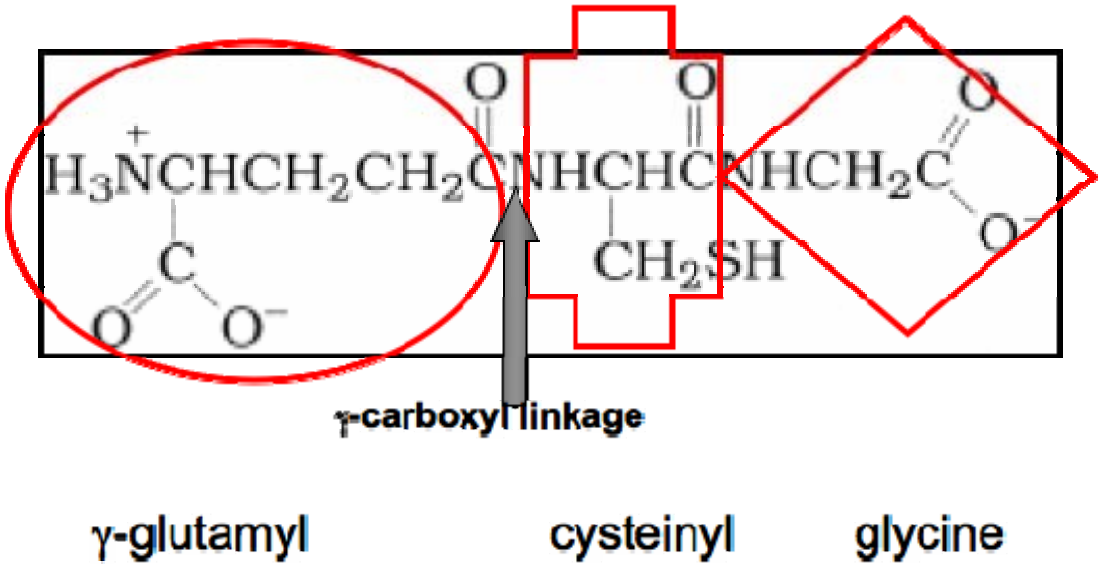

Figure 1.1 Structure of glutathione. The amino terminal glutamate and cysteine are linked by the γ-carboxyl group of glutamate.

Because of its low redox potential, GSH directly scavenges hydroxyl and carbon-centered radicals, hypochlorous acid, peroxynitrite, reactive aldehydes, and singlet oxygen (55-57). Aside from directly scavenging oxidants, GSH is also used enzymatically by GSH peroxidases (Gpx) as a co-substrate for the reduction of H_2O_2, alkyl and fatty acid peroxides (33, 58). The product of GSH oxidation by glutathione peroxidase is glutathione disulfide (GSSG). GSSG can be enzymatically converted back into GSH by GSSG reductase (GR) using NADPH as a reductant. Free GSSG can be excreted from cells either by specific transporters (59-63) or by gluthathionylation (direct conjugation to proteins) (57, 64-66). Thus, the GSH/GSSG redox couple, by virtue of being the most abundant in the cell, acts to maintain cellular redox environment (67). Additionally, GSH conjugates to a variety of xenobiotics, mutagens and drugs, aiding in their detoxication by reducing reactivity of toxins and also causing increased excretion from cells (52). Enzymes called glutathione S-transferases (GSTs) mediate conjugation of GSH to drugs and xenobiotics (32, 52, 53), depleting intracellular GSH levels as a consequence. Compounds detoxified by GSTs include reactive aldehydes, chloroform, organic nitrates, and many drugs including acetaminophen (32, 52, 53). The resulting GSH conjugates are much more soluble than the original compounds, leading to their transport across cell membranes and excretion. Thus, GSH is a central mediator in overall cellular defenses against reactive oxygen species as well as xenobiotic, mutagen and drug metabolism.

Compared to all other organs, the liver contains a correspondingly high level of GSH (10 mM) and GSH-related enzymes (68, 69). This is because the liver is the

central organ that clears toxins and xenobiotics. However, the presence of large amounts of toxins can rapidly decrease hepatic GSH, impairing GSH-dependent detoxication and increasing the likelihood of toxic insult. Thus, the GSH detoxication capacity of the liver is dependent on the rate of GSH synthesis as well as the rate of utilization.

Intracellular hepatic GSH levels are regulated by a complex series of mechanisms that include substrate (mainly cysteine) transport and availability, rates of synthesis and regeneration, GSH utilization and GSH efflux to extracellular compartments (52). GSH is synthesized in the cytosol of all mammalian cells from glutamate, cysteine, and glycine in a tightly regulated manner (52, 70). The synthesis is carried out by the consecutive action of two ATP-dependent enzymes (Figure 1.2). γ-Glutamyl cysteine ligase (GCL) catalyzes the first and rate-limiting step of GSH biosynthesis by forming γ-glutamylcysteine from glutamate and cysteine. The γ-peptide linkage formed during the first step protects GSH from degradation by aminopeptidases (Figure 1.1) (54). GCL is composed of a heavy or catalytic (GCLC, 73 kD) and a light or modulatory (GCLM, 30 kD) subunit, which are encoded by separate genes in humans, rats, and mice (71-74). GCL can be dissociated into its two subunits under non-denaturing conditions (75). Although all the catalytic activity is contained within GCLC, GCLM plays an important regulatory role by decreasing the K_M of the holoenzyme for glutamate and increasing the K_i for glutathione. GCL in humans, rats and mice is regulated physiologically by competitive

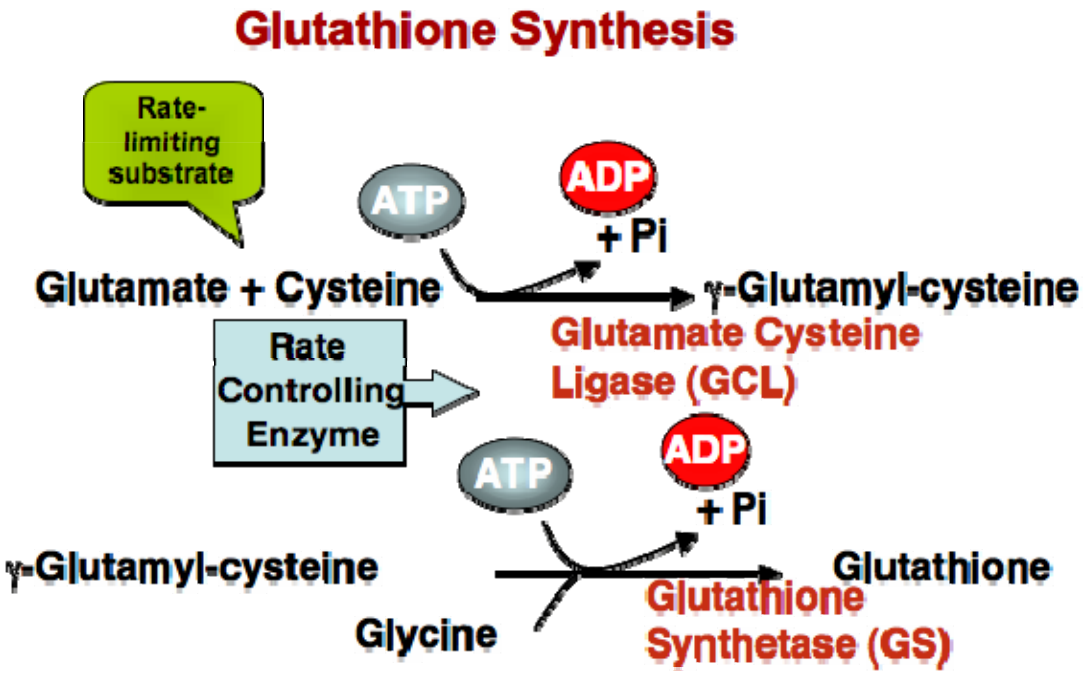

Figure 1.2 Synthesis of glutathione (GSH). GSH is synthesized from glutamate, cysteine and glycine by the sequential actions of glutamate cysteine ligase (GCL) and glutathione synthase (GS). Both reactions require ATP. Cysteine is the rate-limiting substrate in GSH synthesis, while GCL is the rate-limiting enzyme.

feedback inhibition through GSH (76-78), availablity of intracellular cysteine (77, 78), and by transcription of the enzyme subunits (79-83). The second step in the synthesis of GSH is catalyzed by GSH synthetase (GS, 118 kD), which is composed of two identical subunits (84). GS is not subject to feedback inhibition by GSH and is dispensable under both normal and oxidative stress conditions, due to an accumulation of γ-glutamylcysteine, which protects against oxidative stress (85). Furthermore, over-expression of GCL, but not GS increased GSH levels in yeast, underlining that GCL is the rate-limiting enzyme in GSH synthesis (85).

In addition to synthesis, GSH can be transported out of cells, thereby providing plasma GSH and GSSG. The extracellular, membrane-bound enzyme γ-glutamyl transferase (GGT) is the only enzyme which can break the γ-peptidyl linkage (52). GGT can either transfer the γ-glutamyl group to amino acid acceptors to give γ-glutamyl peptides and cysteinylglycine or directly hydrolyze GSH to glutamate and cysteinylglycine (52). Cysteinylglycine can be cleaved by a dipeptidase and the free amino acids can be transported back into cells to be used in the regeneration of GSH. Thus, GSH levels are tightly regulated within cells.

During aging, GSH concentrations decline, thereby putting cells at increased risk of succumbing to stress, and this change has provided a consistent piece of evidence supporting the concept that oxidative stress increases with aging. Numerous studies have shown so far that constituent GSH levels are lower with age in the cerebral cortex, heart, kidney, liver, lungs, lens, skeletal muscles, red blood cells

(RBCs), and testis of various species of rats, mice and humans (9, 10, 86-101). More importantly, there is a significant age-associated decline in GSH concentrations in the plasma, RBCs, muscles and lens of humans (102, 103). The underlying mechanism of age-related GSH loss, however, is not clear. As described above, changes in GSH concentration can occur because of variations in transport and availability of precursor amino acids, changes in rates of synthesis and regeneration, as well as GSH utilization and efflux to extracellular compartment. In the past few years, many laboratories have systematically explored the mechanisms underlying the age-associated decline in GSH levels. Results showed that the activities of GGT and GR were not significantly different between aged and young rats (51, 104-109). Additionally, there was no significant age-associated decline in cysteine (94, 95, 110), or an age-associated increase in GSSG (94, 104, 110), especially in the liver. Glutathionylation of proteins was found to increase in the aging liver, but despite this increase, protein-bound GSH levels could not account for the extent of age-related GSH loss (56, 94). These data suggest that the age-associated decline in GSH content is not caused by increased oxidative stress or decreased availability of cysteine. Recently, many studies examining the rate of *de novo* synthesis of hepatic GSH in old animals have shown a decrease in activity and levels of GCL (51, 94, 104, 111, 112).

Age-related loss in GCL transcription

The activity of GCL is regulated at the transcriptional, translational and post-translational levels and is a complex interplay of these different levels of regulation (53, 113, 114). For example, GCLC and GCLM do not exist in equimolar ratios in

resting cells, with GCLC far in excess of GCLM (115). During stress, the ratio of GCLC:GCLM becomes smaller, although the absolute molar amount of GCLC remains greater than that of GCLM. This suggests that GCLC alone is responsible for the constitutive synthesis of GSH, whereas the association of GCLM with GCLC is needed for higher levels of synthesis such as those required during the response to stress, presumably to overcome the feedback inhibition of GSH (115). Accordingly, mice deficient in GCLC die at an early stage of embryogenesis whereas mice deficient in GCLM survive to maturity, but show a significantly enhanced sensitivity to stress (116).

The *Gclc* gene is encoded by a single-copy gene in the haploid human and rat genome (115). The 5'-untranslated regions for *Gclc* in humans and mice have been cloned, sequenced, and analyzed for regulatory elements that could mediate inducible transcription (79, 80, 115). A number of cis- factors are implicated in the transcriptional activation of *Gclc* mRNAs including activator protein 1 (AP-1) or TPA-responsive element (TRE), AP-2, NF-kB, SP-1 and the antioxidant response element (ARE) (53, 113, 114). However, the sequence of the rat promoter for *Gclc* has only been partially analyzed, presenting a significant gap in understanding the transcriptional regulation of *Gclc* during aging using rat models (80, 81). Still, computational analysis of 5 kb of the rat *Gclc* promoter reveals that the cis-acting elements discovered in the human *Gclc* 5'-flanking region are well conserved in the rat (Figure 1.3).

Of the above-mentioned enhancer elements, those that have received the most

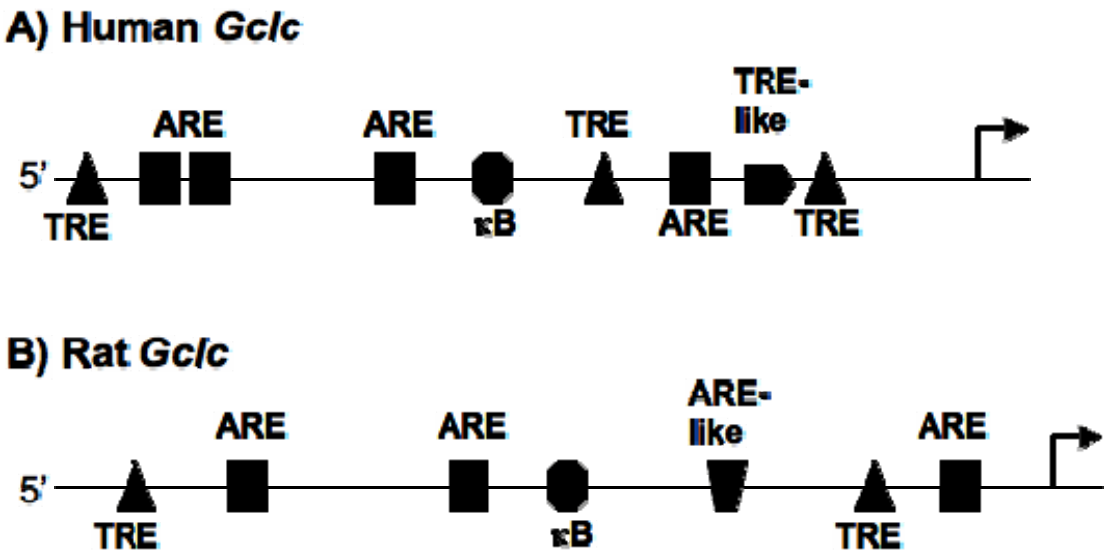

Figure 1.3 Diagrammatic representation and comparison of the human and rat *Gclc* promoters. (A) Human *Gclc* promoter depicting the relative locations of selected enhancer elements. The proximal promoter contains a TRE-like (pentagon) motif, several TREs (triangle), and an NFκB site (octagon) (117). Most notable are the four ARE binding sites (squares). Only the most distal ARE (left), referred to as ARE4 (at -3491), is effective in mediating transcription. (B) The rat *Gclc* promoter showing the relative location of AREs and TREs. The TRE sites are located at the same position in the rat promoter as the human promoter (A), but there is an upstream ARE element located at -865 bp. Additionally, there are only three ARE elements in the rat *Gclc* 5'-flanking region compared to 4 in the human promoter. However, the rat promoter has the presence of an additional ARE-like element at -2107 bp.

attention have been TRE or TRE-like elements and AREs.

The antioxidant response element

The ARE regulates over a hundred genes important for xenobiotic metabolism and antioxidant defense (83, 118-123). The consensus ARE sequence was initially characterized by Rushmore et al. (124) and was described as containing two or more perfect or imperfect TRE binding sequences. This group showed that the DNA sequence, 5'-rTGACnnnGC-3' where 'r' represents a purine, was essential for ARE activity. This minimal consensus element contains one TRE-like element (TGAC) and one GC box (GCA). The ARE consensus element was subsequently revised and extended by Wasserman and Fahl (125). Currently, it is thought that essential cis-acting sequences must be present in the sequences flanking the ARE core. Rushmore and colleagues refined the ARE as being 5'-TMAnnRTGAYnnnGCRwwww-3', where M represents adenine or cytosine, R represents adenine or guanine, Y represents cytosine or thymine, w represents adenine or thymine, and n represents guanine or cytosine (124, 126, 127). Based on this refinement, AREs can be roughly divided into three classes in which the level of ARE transcriptional activity is fairly predictable based on the presence or absence of specific ARE domains (125). These include: 1) class I AREs (5'-TGACnnnGC-3'), 2) class II AREs (5'-RTGAYnnnGCR-3'), and 3) class III AREs (5'-TMAnnRTGAYnnnGCRwwww-3'). Class III AREs include the primary core plus the refined flanking consensus sequences. This class of AREs has high basal activity and is also 2.5 to 50-fold inducible under stress. Class II AREs

contain only the flanking 'R' nucleotides along with the primary core and are only 2.5 to 5-fold inducible. On the other hand, class I AREs contain only the primary core and have very weak basal and inducible transcriptional activity.

Mulcahy and co-workers cloned the 4 kb upstream 5'-flanking region of human *Gclc* (117). Using site-directed mutagenesis, they reported that a distal ARE, which they termed ARE4, is responsible for the constitutive expression of *Gclc*. Interestingly, this ARE is a class III ARE exhibiting high levels of basal and inducible transcription of Gclc (79, 114, 117, 128-130). Thus, it has unequivocally been shown that the ARE regulates the basal and inducible transcription of *Gclc* in humans as well as mice (131).

However, there is a significant gap in knowledge regarding the role of the ARE in the transcription of rat *Gclc*. This is particularly important in the context that numerous studies documenting age-related loss of GCLC have been performed in the rat model (51, 94, 95, 132). In deciphering the cis-regulatory element responsible for the basal transcriptional regulation of rat *Gclc*, there are differences of opinions and in some cases, even controversies (80, 81, 94, 133-135). Lu and co-workers reported that basal as well as inducible transcription of rat *Gclc* is regulated by an AP1 element located 450 bp upstream from the transcriptional start site (80). This study also did not identify any ARE in the rat *Gclc* promoter region. Subsequent studies including one from the same group reported that the NFE2-related factor 2 (Nrf2) that regulates gene expression through the ARE is essential for rat *Gclc* transcription (133-135). In

addition, it was reported that Nrf2 regulated the transcription of rat *Gclc* indirectly through the AP1 site (135). However, there are two significant caveats in these studies. First, it is important to recognize that this group analyzed only the first 2 kb upstream of the transcriptional start site, so the potential role of distal elements such as ARE3 (Figure 1.3 B) was not assessed (80). Secondly, the rat *Gclc* promoter lacking the distal ARE elements was analyzed in fibroblasts of Nrf2 null mice, hence an accurate mechanism was not obtained (135). Nevertheless, the mechanism of transcriptional regulation of Gclc remains an unsolved problem.

Despite these caveats, the role of AP1 elements in rat *Gclc* transcription cannot be disregarded. ARE sequences often contain embedded AP1 (TRE) binding sites or are flanked by these sequences (136). For example, human *Gclc* ARE4 contains an embedded AP1 site that is required for its basal as well as β-napthoflavone-induced transcription (130). Similarly, the rat *Gclc* ARE3 contains a 5'-flanking TRE sequence, whose role in transcription remains to be explored (Figure 1.3 B).

The ARE bears a remarkable sequence similarity to NF-E2 binding sites and TRE-type Maf Recognition elements (T-MARE) (137, 138). Consequently, ARE sequences may be recognized by basic leucine zipper (b-Zip) transcription factors which commonly bind to Maf Recognition Element (MARE) sequences such as AP1 family members (Jun, Fos, Fra), NF-E2 family (Nrf1, Nrf2) and small maf families (139-145). The relatively large number of potential bZip homo- and heterodimer combinations contributes to a relatively flexible system. Even in the case of *Gclc*,

these combinations of factors could account for a broad range of transcription factor upregulating gene expression through a common cis element.

Transcription Factors Binding the ARE

Nrf2

Studies from many laboratories have conclusively shown that the transcription factor NFE2-related factor 2 (Nrf2) is the main bZip factor binding to the ARE (145-147). For example, gene expression of Gclc was markedly lower in the lung of Nrf2-/- mice (148). Nrf2 belongs to the cap'-n'-collar (CNC) family of transcription factors that share a highly conserved basic region-leucine zipper structure (149). Transcriptional activation of ARE-regulated genes via Nrf2 is regulated at many levels. Under basal homeostatic conditions, Nrf2 is bound to a cysteine-rich protein Keap1 in the cytosol. Keap1 bridges Nrf2 to Cul3, a ubiquitin ligase for degradation and sequesters the molecule from nuclei, preventing Nrf2 from activating target genes (150). In the absence of Keap1, Nrf2 constitutively accumulates in the nucleus, indicating that Keap1 negatively regulates Nrf2 by enhancing its rate of degradation and also altering its subcellular localization (146). Two of the cysteines in Keap1 have been demonstrated to be essential for ubiquitination (48). Based on these observations, several laboratories have demonstrated that Nrf2 is rapidly turned over by the proteasome (118, 151-154). A schematic representation of Nrf2 activation is shown in Figure 1.4.

Nrf2 activity can also be modulated by its heterodimer partner in DNA

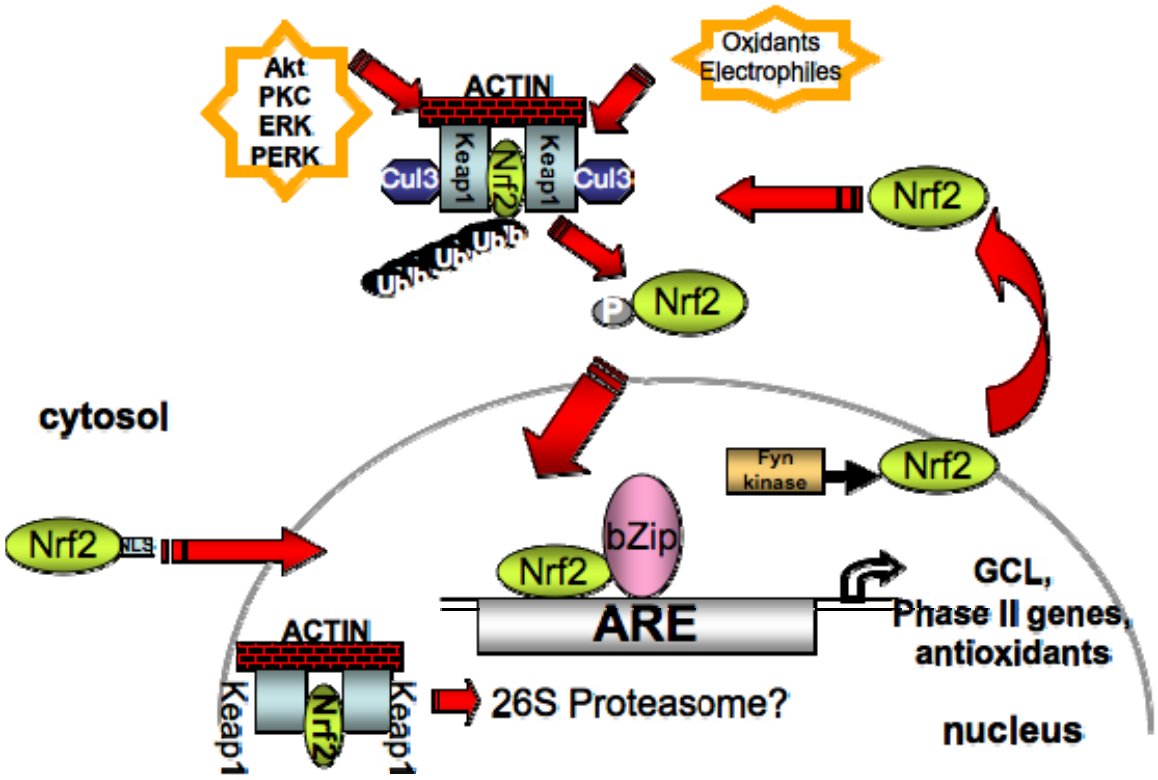

Figure 1.4 Schematic illustration of the molecular mechanisms of the Nrf2–Keap1 regulatory system and its endogenous activating signals. In unstimulated conditions, Nrf2 is tethered onto actin fibers in the cytoplasm through Keap1 and degraded by proteasomes. Thus, the transcription levels of Nrf2 target genes remain low. Upon exposure to electrophiles and/or oxidative stress (ROS, reactive oxygen species), Nrf2 translocates into nuclei, heterodimerizes with a bZip protein and binds to the ARE, leading to the transcriptional activation of Phase II enzyme genes and antioxidant stress protein genes. Keap1 interacts with Cullin 3 (Cul3), one of the components of ubiquitin ligase. These reagents seem to attack the sulfhydryl group of Keap1 and interfere with the interaction between Nrf2 and Keap1 or with the integrity of Keap1 and the ubiquitination machinery, resulting in the release of Nrf2 from Keap1 or the shutdown of Nrf2 degradation.

binding. Nrf2 is not known to bind the ARE independently, but dimerizes with small

Maf proteins, Jun-Fos family members and ATF4 (145, 146, 155-159). The DNA-binding specificities, transcription activation potential and responsiveness to inducers of these distinct dimeric species are likely to vary (see Figure 1.5). Therefore, although a role for Nrf2 in upregulation of ARE mediated gene expression and induction is very well established, the search is still on to find heterodimeric partners of Nrf2 and how they modify transcription.

Other transcription factors

Nrf2 requires a member of the small Maf proteins as an obligatory partner molecule for binding to its cognate DNA sequence (160). It should be noted that the small Maf proteins do not possess a canonical *trans*-activation domain and homodimers of the small Maf proteins act as direct transcriptional repressors (141, 143, 161). The small Maf proteins enable Nrf2 to bind to DNA and exert its function. Thus different biological activities, all elicited through sometimes slightly different MARE binding sites, may come into play depending on the partner molecules with which the small Maf proteins heterodimerize. It has been demonstrated that regulation from ARE sites can be turned on and off in living cells by experimentally manipulating the balance between the small Maf proteins and Nrf2 (161, 162). If the abundance of Nrf2 partner molecules is inadequate to 'titrate' all of the small Maf proteins produced in a cell, homodimers of the small Mafs would then be predicted to form a dominant effect, leading to silencing ARE-driven transcription (163).

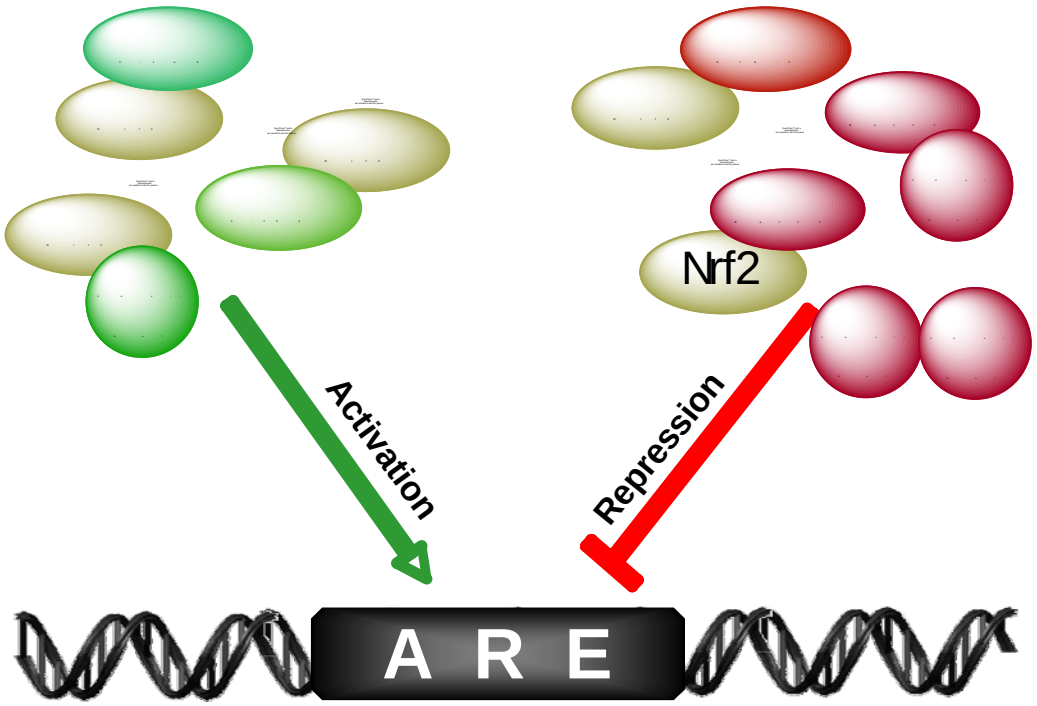

Figure 1.5 Regulation of Nrf2-ARE binding by Nrf2 heterodimerization partners. The consensus sequence of ARE resembles the binding site for many bZip transcription factors. Heterodimers composed of CNC members, including Nrf2 and small Maf proteins, including MafG, MafK and MafF, interact with these elements. Bach1 can also heterodimerize with small Maf proteins. Since the ARE site resembles a TRE or has an embedded TRE element, Nrf2 can also form heterodimer combinations with the Jun, Fos and Fra proteins.

In addition to small Maf family members, new partner molecules of Nrf2 have recently been described which play an even more prominent role in modulating chromatin activity through the ARE element. The Bach family proteins (Bach1 and Bach 2) bind to the ARE consensus sequence as repressive heterodimers with small Maf proteins and consequently, displace Nrf2 (144). Upon activating stimuli, Bach1 is replaced by Nrf2, suggesting competition between Bach1 and Nrf2 for the same binding site in different cellular states (164). Thus the balance between Nrf2 and Bach1 on the target ARE sequence determines the level of gene expression (165). While Bach1 is implicated in the transcription of certain antioxidant and detoxification genes like hemeoxygenase 1 and NAD(P)H: quinone oxidoreductase 1 (164, 166), whether it plays a role in GCLC regulation, is currently unknown.

Certain co-activators with histone acetyltransferase activity also modulate Nrf2 binding to the ARE. Nrf2 contains two activation domains, Neh4 and Neh5, both of which are conserved in various Nrf2 proteins in several species (158). Both Neh4 and Neh5 can bind to the coactivator CBP [cAMP-response-element binding protein (CREB) binding protein] independently, and simultaneous binding of CBP to these two domains synergistically activates the transcription of Nrf2 target genes (158, 167, 168). It is hypothesized that Nrf2 achieves strong transactivation, at least in part, through this mechanism (168). The investigation of CBP in age-related loss of Gclc transcription is particularly attractive in this dissertation, as hepatic CBP activity and levels are documented to decline with age (169).

A final partner molecule with which Nrf2 is known to associate through traditional protein–protein interactions is c-Fos, which has been reported to heterodimerize with small Mafs and hence bind to the ARE consensus sequence, however, the c-Fos–small Maf heterodimeric complex is unable to activate transcription through that site (143). Since all ARE elements are also AP-1 binding sites, an increase in ARE activity could result in a direct competition between AP-1 (Jun/Fos) and Nrf2/Maf for the site. Alternatively, induction of c-Fos alone could lead to sequestration of small Mafs away from their Nrf2, thus providing another avenue to inhibit small Nrf2/small maf formation and activity (44).

Lipoic Acid

Lipoic acid (LA; 1,2-dithiolane-3-pentanoic acid) is a naturally occurring dithiol compound found in high abundance in green leafy vegetables (spinach, kale and broccoli) and meats (170). Though LA is synthesized *de novo* from octanoic acid in mammals (171), dietary consumption may also be necessary to maintain adequate cellular levels. LA is an essential cofactor for mitochondrial bioenergetic enzymes (171, 172). Concentrations of free LA are very low *in vivo* (173, 174). However, following a meal or dietary supplementation, there is a marked, yet transient increase in unbound LA in all tissues studied, including the liver, heart and brain (175, 176). Thus, nonprotein-bound LA only transiently appears in cells (177). Orally administered LA, generally in its oxidized form, is readily taken up into cells and

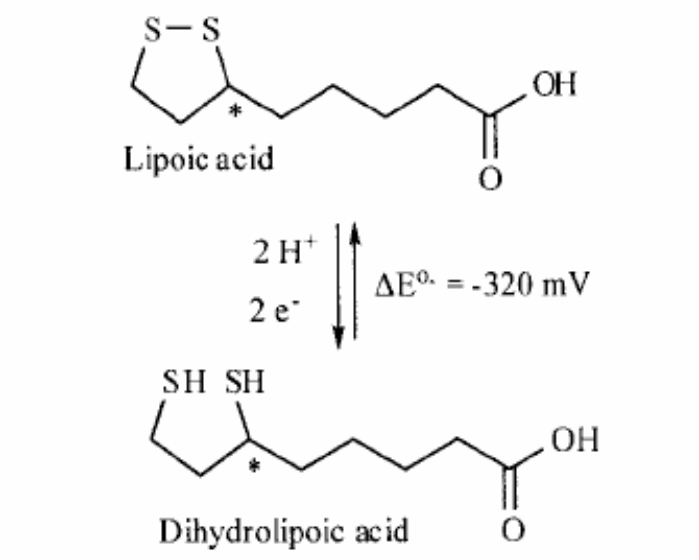

Figure 1.6 Lipoic Acid Structure and Chemical Properties. Lipoic acid (1,2-dithiolane 3-pentanoic acid) is an eight-carbon dithiol compound with a high reduction potential. It has one chiral center that is denoted by an asterisk.

reduced either by mitochondrial lipoic acid dehydrogenases or thioredoxin/glutaredoxin reductases to dihydrolipoic acid (DHLA) (173). This reduction occurs at the expense of NAD(P)H, the only known biological compound with sufficient power to reduce oxidized LA.

LA and its reduced form dihydrolipoic acid (DHLA) have one chiral center (Figure 1.6). The *R*- form of LA is the only enantiomer synthesized and used in biological systems as a prosthetic group for mitochondrial α-ketoacid dehydrogenases. In mammals, LA is synthesized de novo in mitochodria by lipoic acid synthase, but it can also be absorbed from the diet (178-180). The chemical reactivity of LA and DHLA is mainly centered in its dithiolane ring (Figure 1.6). Torsional strain distorts this ring and contributes to the reactivity of both thiol groups (181, 182). These structural features make LA a highly reactive molecule under physiological conditions, which is evident in a DHLA/LA reduction potential of -0.32V (183, 184) (Figure 1.6). Consequently DHLA is capable of reducing a host of other compounds including disulfides, the oxidized forms of antioxidants (GSH, vitamins C and E) and transition metal ions (185, 186).

There is no question that LA treatment boosts cellular GSH levels both in vitro and in vivo in times of oxidative stress (187, 188). Aside from a direct reduction of GSSG to GSH, LA also induces GSH synthesis either by increasing cysteine availability (95, 189) and/or possibly by enhancing transcription of GCL (94). More specifically, the dithiol nature of LA could activate the Nrf2-Keap1 system through reaction with cysteines present in Keap1. Alternatively, LA can activate potential

upstream kinases involved in the activation of Nrf2. This gives a good rationale for use of LA to boost the decreased GSH levels during aging.

1.1 Dissertation hypotheses and aims

Despite substantial literature precedent documenting the loss of GSH with age and how it adversely affects stress resistance in the elderly, no significant attempt has been made to understand the underlying mechanisms of age-related GSH deficiency. Considering that boosting GSH levels would have a high payoff in maintaining elder health, design of suitable prophylactic therapies to maintain GSH homeostasis would require a more detailed understanding of why GSH levels fail with age. We contend that lipoic acid is one potential therapeutic agent to maintain GSH levels that otherwise decline with age. In this regard, we further suggest that LA may act as a nutritive key to unlock any alterations occurring either during the synthesis of glutathione or transcriptional control of the synthesis itself.

Based on our contentions, this dissertation can be divided into two broad hypotheses. The first hypothesis is that **age-related decline in glutathione levels is due to a lesion in the transcriptional control of its synthesis.** This hypothesis has been examined by addressing the following questions:

1) Is GSH synthesis impaired with age? If so, do GSH-synthesizing enzymes decline in the elderly? This question will be answered using liver tissue of young and old Fischer 344 rats, which is a good model of aging.
2) Is there an age-related lesion in the Nrf2/ARE transcriptional network governing Phase II genes? Is this network responsible for any alterations in GSH synthesis during aging? These questions will be answered using a novel

primary hepatocyte cell culture model designed to study aging. The end-points measured will be:

a) Steady-state nuclear availability of Nrf2 in young and aged rats
b) Alterations in Nrf2 and partner bZip transcription factor status at the ARE transcriptome

Secondly, we hypothesize that **lipoic acid treatment reverses the age-related loss in GSH synthesis by replenishing nuclear Nrf2 availability and binding to the antioxidant response element.** This hypothesis has been explored by addressing the following questions:

1) Does LA treatment reverse the age-related loss in GSH-synthesizing enzymes?
2) Does LA treatment replenish nuclear Nrf2 lost during aging?
3) Lastly, what is the effect of LA on the ARE transcription complex that is impaired with aging? This will elucidate the exact mechanism by which LA restores transcriptional activity of GSH synthetic genes.

Chapter 2

Transcriptional Regulation of Rat γ-Glutamate Cysteine Ligase Catalytic Subunit Gene: Role of Antioxidant Response Element 3

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2.1 Abstract

The transcriptional regulation of rat γ-glutamate cysteine ligase catalytic subunit (GCLC) is currently controversial. Computer-based sequence analysis of the gene encoding rat GCLC identified three putative antioxidant response elements (AREs) within the region at positions –889 to –865 (ARE1), -3170 to -3146 (ARE2) and –3901 to –3877 (ARE3). Transfections of individual ARE-luciferase reporter gene constructs into H4IIE cells, a rat hepatoma cell line, resulted in identification of ARE3 as the functional ARE. Chromatin immunoprecipitation showed that only endogenous ARE3 constitutively associated with the transcription factor Nrf2, which is known to regulate ARE-mediated gene expression. Co-transfection of H4IIE cells with *GCLC* ARE3- luciferase reporter plasmids and an Nrf2 expression plasmid resulted in a 3-fold activation of *GCLC* ARE3-mediated transcription relative to controls. "Loss-of-function" analysis for Nrf2 conducted by small interfering RNA (siRNA) revealed that ARE3-mediated expression was significantly impaired. Finally, site-directed mutagenesis of the ARE3-luciferase reporter plasmid abolished Nrf2-mediated induction. Taken together, these results show that Nrf2 regulates the constitutive expression of rat glutmate cysteine ligase catalytic subunit gene through a distal ARE present in its 5'-flanking region. This is the first report that acknowledges the rat *Gclc* gene under the transcriptional control of the Nrf2-ARE pathway on a constitutive basis.

2.2 Introduction

Glutamate cysteine ligase (GCL) catalyzes the first and rate-limiting step of the *de novo* synthesis of glutathione (GSH), the most abundant non-protein thiol in the cell (190). GSH plays key roles in detoxifying peroxides, electrophiles, and maintaining the normal intracellular redox status (14, 52, 54, 67). The *de novo* synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and subsequent formation of GSH from γ-glutamylcysteine and glycine (53, 191). GCL is the rate-controlling enzyme for GSH synthesis (192). The GCL protein is a heterodimer that can be dissociated under non-denaturing conditions into a catalytic (GCLC, 73 kDa) and a regulatory or modulatory (GCLM, 29 kDa) subunit, which are encoded by separate genes (73). Although the heavy subunit contains the entire catalytic activity, association with GCLM can modulate its activity (192). Since GCL is a major determinant of the overall capacity of GSH synthesis, regulation of GCL subunits has been a topic of extensive research (191). Changes in GCL activity can result from regulation at multiple levels affecting only the catalytic or modifier subunits or both. In addition, the enzyme can be regulated at kinetic, post-translational and transcriptional levels (191, 193, 194). An example of the first regulatory mechanism is the feedback inhibition of GCL activity by GSH; indeed, a decrease in GSH will cause a transient elevation of GSH synthesis due to less feedback inhibition on the activity of pre-existent GCL (75, 195, 196). However, the regulation of GCL at the transcription level produces a more persistent effect and thus is more important for

the maintenance of GSH homeostasis in response to oxidative stress (197). It has been well established that both GCLC and GCLM are inducible at the transcription level by various agents such as quinones (*tert*-butyl hydroquinone), dithiolethiones (anetholedithione), isothiocyanates (sulforaphane), and dithiols (lipoic acid) (113).

The distal 5'-flanking region of *Gclc* has been fully sequenced in the human (117) and mouse (82), but not yet in the rat (80). Several DNA *cis*-elements including those for NF-κB, the AP-1 binding site, and the antioxidant response element (ARE) have been implicated in GCL gene regulation (114). In humans and mice, the ARE (core sequence: GTGACNNNGC) has been identified as the regulatory component responsible for the induction of *Gclc* both on a constitutive basis and in response to oxidative or electrophilic stress (79, 115, 117, 147). The 5'-flanking region of rat *Gclc* has only been partially characterized (80, 133, 135, 198-200). Only a 1.8 kb sequence upstream of the start site has been analyzed, and several binding sites for AP-1 or NFkB were reported (80). Yang and co-workers (133) suggested that an AP-1 sequence in the proximal promoter region of *Gclc* is critical to its transcriptional upregulation in response to oxidative stress. In part, this contention is because AREs that are present in the human *Gclc* promoter, are not found in this 1.8 kb region of the rat *Gclc* promoter. However, ARE binding activity has been detected using an ARE4-containing sequence from the human GCLC gene (201). A 44-bp ARE sequence, which shares a 31 bp sequence with the human *Gclc* ARE, has been recently designated as the rat *Gclc* ARE, but no additional sequence information has been disclosed (157). Thus it is possible that functional AREs in the rat GCLC gene may be

present further upstream of the known 5'-flanking regions. Nonetheless, which cis-acting element is involved in the basal gene expression of rat GCLC is still unknown. Despite recent progress in identifying ARE-regulated genes and understanding functional mechanisms of transcriptional regulation, whether the rat GCLC is regulated by an ARE, is still a matter of controversy.

Many transcription factors have been reported to bind ARE, such as Nrf2 family members (Nrf1/2/3), small maf proteins (maf G/K/F), Jun (c-Jun, JunB, JunD), and Fos family members (c-Fos, FosB, Fra1, Fra2) (reviewed by Jaiswal) (121). Among them, ARE-dependent GCLC gene expression is largely dependent upon Nrf2, a member of the Cap'n'Collar (CNC) family of bZIP proteins (79, 202). Nrf2 is located primarily in the cytosol but upon stimulation, accumulates in the nucleus, where it heterodimerizes with other leucine zipper proteins (e.g. c-Jun and small maf proteins), and binds ARE to initiate gene transcription (146, 160). Importantly, when Nrf2 is overexpressed in cells by transfection, Nrf2 accumulates in the nucleus and activates transcription (203, 204).

The aim of the present study was to determine whether Nrf2-controlled basal transcriptional regulation of rat *Gclc* through an ARE-dependent mechanism. Three putative AREs were identified in the promoter region of the rat GCLC gene. Further studies revealed that the ARE (ARE3) present at about 3.9 kb upstream of the transcription start site in the *Gclc* promoter had maximum transcriptional activity compared to the other ARE and AP1 *Gclc* promoter elements. Chromatin

immunoprecipitation (ChIP) assays demonstrated binding of Nrf2 with rat *Gclc* ARE3. Treatment of H4IIE cells with Nrf2-specific RNAi significantly reduced Nrf2 gene expression and ARE3-mediated basal transcription. The present evidence represents, for the first time, that the rat *Gclc* is under basal control of an ARE.

2.3 Materials and Methods

Chemicals and antibodies

Restriction enzymes and T4 DNA ligase for subcloning were from New England Biolabs (Boston, MA). The dual-luciferase reporter assay system and reporter plasmids, pGL4 minimal promoter vector and phRL-CMV vector were from Promega (Madison ,WI). Expression vector for Nrf2 (pcDNA3.1-Nrf2) was a kind gift provided by Dr. Jaiswal at Baylor College of Medicine, Houston, Texas (205). Custom oligonucleotides used in PCR cloning, subcloning, and DNA sequencing were purchased from Invitrogen (Carlsbad, CA). Sequence service was provided by Center for Gene Research and Biocomputing at Oregon State University. Rabbit anti-Nrf2 (H-300) antibody, anti-lamin B1 antibody and Nrf2 siRNA and scrambled sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin antibody was from Sigma-Aldrich. All chemicals used were at least analytical grade.

Animals

Male Fischer 344 rats were purchased from the National Institute of Aging colonies. The rats were maintained in separate security barriers operated by Lab Animal Resources, Oregon State University according to the guidelines established in the Guide for the Care and Use of Laboratory Animals (Animal Resources Commission on Life Sciences, National Research Council) and under specific pathogen free (SPF) conditions.

Identification of ARE(s) in the promoter of rat GCLC

To identify the presence and location of putative ARE(s) in the rat *Gclc* promoter, the 5 kb upstream from the translation start site was downloaded from the NCBI database (www.ncbi.nlm.nih.gov/genome/guide/rat). This sequence was used to search for putative ARE(s) with the help of the MatInspector (206) and the Matrix Family Library software (version 2.4; MatInspector, Genomatix, Munich, Germany) using the ARE primary core sequence (RTGAYNNNGCR) as the probe. The location of the transcription start site for the rat GCLC gene has already been determined (80).

Construction of luciferase reporter vectors

The ARE- and AP1-luciferase reporter plasmids were generated using the pGL4-minimal promoter vector (Promega) containing a minimal TATA promoter upstream of the firefly luciferase gene. The sequences of the inserts used in the different plasmids are summarized in Table 2.1. Single-stranded oligonucleotides were first annealed to form double-stranded oligonucleotides and then ligated into the pGL4.23[minP] vector following the manufacturer's instruction (Promega). Each vector was engineered by inserting 3 copies of each of the ARE elements present in the rat *Gclc* 5'-flanking region. The three different *Gclc* 25-bp ARE (3X)-driven luciferase reporter constructs [i.e., *pGL4.23Gclc*-3xARE1-Luc2, *pGL4.23Gclc*-3xARE2-Luc2, and *pGL4.Gclc*-3xARE3Luc2] were made by insertion of the appropriately hybridized complementary oligonucleotides [75 bp] with 2-bp overhangs into the *Xho*I/*HindIII* restriction sites of the pGL4-minimal promoter

reporter vector. TOP10 competent cells were transformed with the recombinant DNA after ligation for amplification. After the plasmids were generated, the DNA sequence of the inserts was verified.

Cell culture, transfections and luciferase assays

Rat hepatoma-derived H4IIE cells, obtained from American Type Culture Collection (Rockville, MD) were grown in MEM supplemented with 10% FBS, 2 mM glutamine, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37 °C in a 5% CO2 atmosphere. Reporter gene assays were used to determine effects of different ARE and AP1 elements on transcriptional activity of the rat GCLC gene. The pGL4 minimal reporter vector contains a genetically engineered firefly luciferase gene containing a minimal promoter. When promoter regions are cloned into the pGL4 vector upstream of the luciferase gene, there is strong transcriptional activation and expression of luciferase. Mutations in the individual promoter elements can influence transcriptional activity of the luciferase reporter gene that is detected fluorometrically *vs.* an internal control. Transient transfections were done in cells grown to ~50% confluence using the Effectene Transfection reagent (Qiagen, Valencia, CA). The cells were transfected with 0.5 μg of *Gclc*-Luciferase plasmids. Briefly, the DNA and 4 μl of Enhancer were dissolved in buffer EC from the kit to a total volume of 100 μl. The DNA–enhancer mixture was incubated at room temperature for 5 min. After incubation, 5 μl of Effectene transfection reagent was added to the mixture, mixed, and incubated at room temperature for 10 min to allow transfection–complex formation. Medium (200 μl) was added to the mixture and

mixed. The mixture was then immediately added to the well containing the cells and 1.5 ml of fresh medium. The total amount of plasmid DNA for transfection was adjusted by empty expression vector (pGL4.23). 0.02 μg of the control plasmid phRL-CMV encoding *Renilla* luciferase was included in each transfection to account for variability in transfection efficiency. Thirty-six hours after transfection, cells were harvested with 1 x passive lysis buffer (Promega), and the supernatant was collected by brief centrifugation. Transcription activity was determined by the expression of firefly luciferase and was normalized to the renilla luciferase levels by using a dual luciferase reporter assay kit (Promega) on a Biolumat LB9505 luminometer (Berthold Detection Systems, Pfhorzeim, Germany). The means of at least three independent experiments, each carried out in duplicate, are shown and expressed as the mean $\pm$ S.E.

Nrf2 siRNA knockdown

Nrf2 siRNA (50 pM) or scrambled control (50 pM) was transfected to H4IIE cells by Effectene (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, cells were seeded in six-well plates and incubated overnight, then transfected with 50 pM siRNA for 12 h using 10 μl Effectene per well. The cells were cultured for another 24 h, then used for experiments. There was almost no visible damage due to the transfection procedure.

RNA isolation and real-time PCR

Total RNA (0.5 mg) was reverse-transcribed with random primers and the

resulting cDNA was amplified by real-time PCR using the MJ Research Opticon 2 (BioRad, Hercules, CA).

Preparation of nuclear extracts and Nrf2 analysis

Cells were scraped into ice-cold lysis buffer (10 mM Hepes, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 10 mg/mL pepstatin A, 0.5 mM DTT, and 0.4% NP-40), incubated for 10 min, and centrifuged at 14,000 g for 3 min at 4 °C. The resulting nuclear pellet was resuspended in extraction buffer (20 mM HEPES (pH 8.0), 0.4 M NaCl, 1.0 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10 mg/mL aprotinin, 10 mg/mL leupeptin, 10 mg/mL pepstatin A, and 10% glycerol), kept on ice for 15 min, and centrifuged at 14,000 g for 5 min at 4 °C. The supernatant containing the nuclear proteins was resolved by SDS-PAGE and Nrf2 levels determined by western blotting using Nrf2 antibody (1:1000).

Western immunoblotting

Cells were lysed in 50 mmol/L Tris–HCl—150 mmol/L NaCl, pH 7.5, buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 100 mmol/L NaF, 2 mmol/L Na3VO4, 10 mmol/L PMSF, 150 nmol/L aprotinin, and 1 mmol/L leupeptin. Protein concentrations were measured with the Folin Lowry Protein Assay. Forty μg of protein were electrophoresed in 10% SDS-PAGE. After transfer to nitrocellulose membranes, bands were visualized by reaction with primary antibody. In brief, membranes were blocked in 5% fat-free milk and probed with primary antibody for 1 h at room temperature. After secondary incubation in horseradish peroxidase-

conjugated donkey anti-rabbit IgG antibody (1:5000) (Amersham, Piscataway, NJ), the immunocomplexes were visualized with an enhanced chemiluminescence western blotting reagents (ECL Plus) from Amersham and resulting autoradiographs were exposed to Hyperfilm (Amersham). Bands corresponding to different proteins were quantified by scanning of photographs and then digitalized and analyzed with the NIH Image software. All cytoplasmic protein was normalized to actin and all nuclear protein was normalized to lamin B1 (1:1000) as loading controls and measured for significance (data not shown). Western blots were run in triplicate. To strip membranes of antibody when necessary, a glycine buffer (pH 2.2) was used followed by blocking and reprobing with the appropriate antibody.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation analysis was conducted using control rabbit IgG, anti-Nrf2 (Santa Cruz; sc-13032) antibodies. PCR primers are described in Table 1. Briefly, proteins and DNA were cross-linked with 1% formaldehyde for 10 minutes at room temperature and cells lysed in SDS-lysis buffer containing protease inhibitors and then sheared to an average length of 500-800 bp by sonication using a Sonic Dismembrator Model F60 (Fisher Scientific, Pittsburgh, PA). Sheared chromatin was immunocleared with salmon sperm DNA/protein A-sepharose and 10% of the precleared chromatin was stored and labeled as "input DNA". The remaining chromatin was immunoprecipated with IgG (control) or Nrf2 antibodies (2 μg) overnight at 4°C. Immune complexes were adsorbed onto salmon sperm DNA/protein A-sepharose beads. Immunoprecipitates were washed sequentially with wash buffers

to reduce background. Protein–DNA complexes were eluted from the antibody with elution buffer (1% SDS, 0.1 M NaHCO3) and formaldehyde cross-links reversed by addition of NaCl (5 M) and heating at 65 °C overnight. DNA was purified using the QIAquick purification kit (Qiagen, Valencia, CA) and PCR performed using primer pairs that spanned each of the ARE and AP1 elements. PCR products were quantified using SYBR Green Mastermix (New England Biolabs) with the Mini Opticon 2 Real-Time PCR Detection System (Bio-Rad). Specific enrichment of DNA by anti-Nrf2 antibody was calculated by subtracting the PCR value of normal IgG from that of anti-Nrf2 antibody and by normalizing that value to the PCR input. Triplicate PCR reactions were conducted for each sample, and the experiments were repeated at least thrice. The specificity of the PCR products was confirmed by melting curve analysis and size (agarose gel electrophoresis).

Statistical Analysis

The data are expressed as the mean $\pm$ S.E. Statistical analysis was performed using GraphPad Prism software version 3.03 (GraphPad Software Inc., San Diego, CA). We used a two-tailed Student's *t* test to compare the luciferase activity of individual *GCLC* promoter constructs. A *P* value less than 0.05 was considered to be significant. One-way analysis of variance (ANOVA) was used when multiple comparisons were made, followed by Tukey's post-hoc analysis for multiple comparisons to a control.

Table 2.1. Sequence of inserts in the pGL4 minimal promoter vector

Plasmid	Sequence of interest (5'→3')
Pgl-3xAP1	ACTGAGTGACC
Pgl-3xARE1	GATTTACAATGACTAGACACACGTAGATTTACAATGACTAGACACACGTA GATTTACAATGACTAGACACACGTA
Pgl-3xARE2	TCCTGACAGTGAGTTGACACTCTGG TCCTGACAGTGAGTTGACACTCTGG TCCTGACAGTGAGTTGACACTCTGG
Pgl-3xARE3	GAGTCACGGTGAGGCGGCACGGCGCGAGTCACGGTGAGGCGGCACGGCGC GAGTCACGGTGAGGCGGCACGGCGC
Pgl-3x(TRE)ARE3	GTTGAGTCACGGTTGAGTCACGGTTGAGTCACG
Pgl-3xARE3	GAGTCACGGGTCGGCGTAACGGCGCGAGTCACGGGTCGGCGTAACGGCGC GAGTCACGGGTCGGCGTAACGGCGC

NOTE: The underlined letters represent those nucleotides that form the minimal functional ARE or AP1 element. The mutated ARE3 core sequence is in bold.

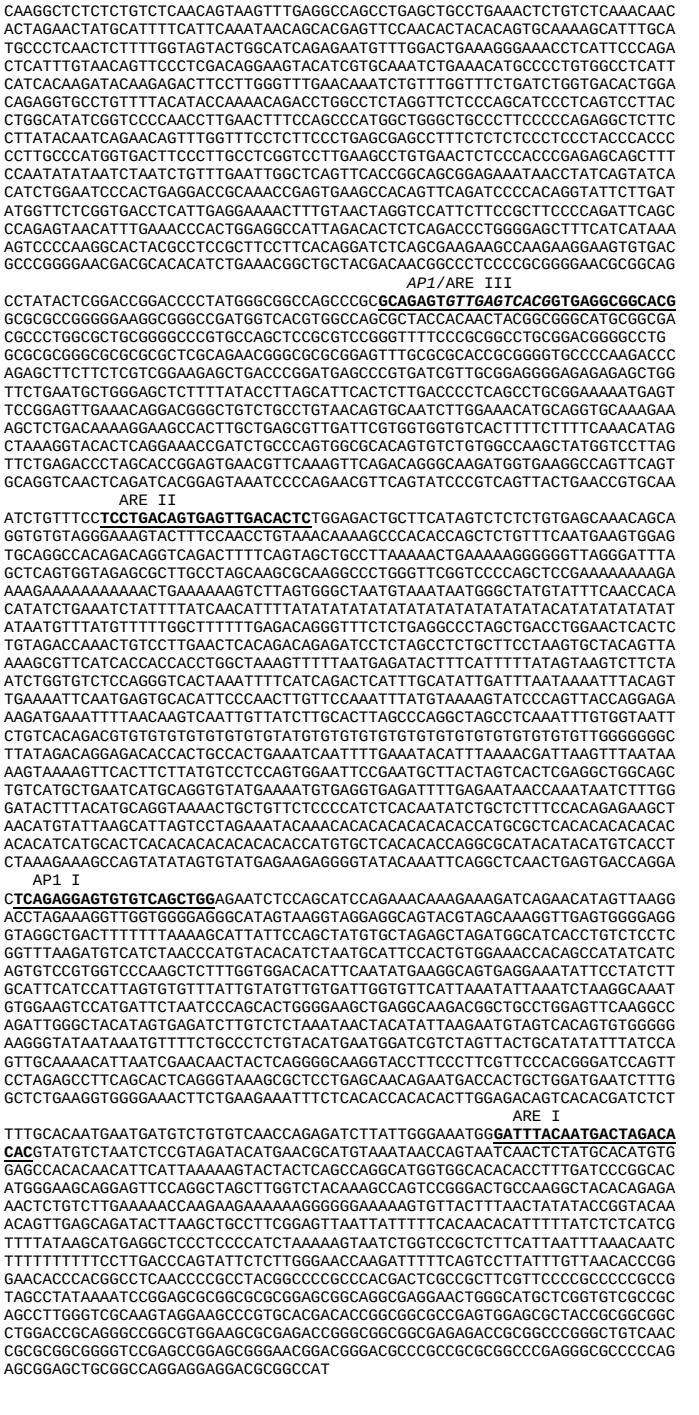
2.4 Results

Identification of a functional ARE in the Gclc promoter

We examined the rat *Gclc* promoter for potential antioxidant response elements (AREs) by a computer-based analysis. Nucleotide sequence analysis using the TRANSFAC database and the ARE consensus sequence as a probe (197) revealed that there are three ARE motifs (designated ARE1-3) within 5 kb of the *Gclc* 5'-flanking region (Figure 2.1A). When compared to the human *Gclc* promoter sequence, ARE3 is identical to human *Gclc* ARE4 while ARE1 contains a single mismatch (117, 128, 130, 207). Identity between the rat and human sequence suggested that the ARE3 site of the rat sequence might be important in *Gclc* transcription. To characterize the contribution of each ARE to the basal transcriptional regulation of *Gclc*, ARE constructs were created by synthesizing three tandem copies of each ARE element and subcloning them into the pGL4 minimal promoter vector followed by transient transfection into H4IIE cells and analysis of luciferase activity (Figure 1B). The results revealed that only ARE3 at nucleotide position –3901 was capable of mediating an increase in basal expression. All other AREs failed to significantly increase basal expression. The ARE3 sequence is identical with the previously reported minimal ARE class III enhancer sequence (a/g)TGA(C/T/G)nnnGC(a/g) (190). In contrast, the ARE2 and ARE3 sequences differ from the consensus sequence in that the 3' "GC" is replaced by "AC".

Previous studies reported that the active ARE in the human *Gclc* (ARE4) also

Figure 2.1 A



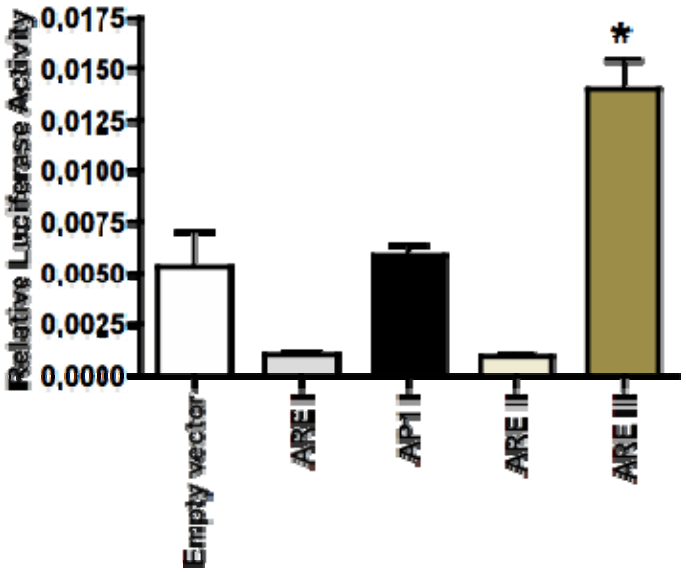


Figure 2.1 B

Figure 2.1 ARE3 regulates constitutive expression of rat *Gclc* (A) Representation of the 5'-flanking region of *Gclc*, which is conserved between human, mouse and rat. The numbers shown underneath represent DNA bases in the 5'-promoter, as counted from the transcriptional start site. The putative ARE and AP1 elements conserved between the three species are shaded. (B) H4IIE cells were transiently transfected with each of the *Gclc*-ARE or *Gclc*-AP1 elements cloned into the pGL4 luciferase reporter with a minimal promoter together with a CMV-Renilla luciferase transcription control plasmid. Luciferase activity was determined in the cytosolic fraction 36 hr after transfection. Results are presented as relative luciferase units (RLUs) normalized to the Renilla luciferase internal control. Statistically significant expression compared with empty vector, respectively, is marked with asterisks: *, $P < 0.05$ ($n = 3$; 1-way ANOVA).

contained an adjacent TRE element and was potentially involved in the induction of the gene (117, 128, 129). Since the ARE consensus sequence resembles the AP-1 binding site and the rat *Gclc* ARE3 has an embedded AP-1 element at its 5'-end, we created an AP1 construct contained within the ARE3 site and transfected it into H4IIE cells. Results revealed that the AP1 component displays 52% of the basal transcriptional activity of the ARE3 site (data not shown).

Nrf2 binds to GCLC ARE3 in an endogenous chromatin configuration

Since Nrf2 is the most potent transcriptional activator among CNC proteins (158, 208-210), Nrf2 may enhance cytoprotective gene expression even if the other transcription factors are present and occupy the ARE. To examine whether each of the three AREs in the 5'-flanking region of *Gclc* has the ability to bind Nrf2 *in vivo*, we determined the binding of Nrf2 to the individual ARE elements in their native chromatin environment by chromatin immunoprecipitation assay (ChIP) analysis. To control for possible nonspecific interactions and DNA contamination, samples precipitated with rabbit immunoglobulin G were included. Primary rat hepatocytes were cross-linked with formaldehyde, and chromatin was immunoprecipitated using an anti-Nrf2 antibody. PCR analysis revealed Nrf2 binding only to the region between 3.2 and 4 kb upstream of the transcriptional start site (Figure 3.1A and 3.2), which harbors the functional ARE3. Thus, our results validated the data we obtained from luciferase reporter activities showing that ARE3 was the only functional element among the three AREs in the rat *Gclc* gene. The recruitment of Nrf2 to these ARE sites was specific because no signal was detected in the immunoglobulin G control

Figure 2.2 Nrf2 binds only to ARE3 *in vivo* (A) Schematic presentation of the primer locations used for ChIP analysis. The rat *Gclc* 5'-flanking region includes three AREs. (B) Nrf2 only binds to *Gclc* ARE3 efficiently. Primary rat hepatocytes were cross-linked with formaldehyde for 10 minutes, and ChIP analysis was performed using antibodies against Nrf2. Normal rabbit IgG was used as a control.

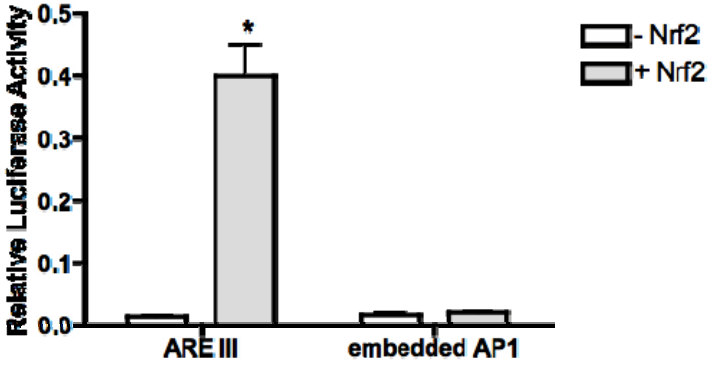
samples. We did not find an increase of Nrf2 recruitment to the nonspecific intervening region located between ARE1 and ARE2. The results obtained from DNA that was PCR amplified from chromatin extracts before immunoprecipitation (input) are shown for comparison. These results thus demonstrate that Nrf2 binds only to ARE3 out of the three putative AREs identified in the 5'-promoter of rat *Gclc*.

Nrf2-dependent upregulation of Gclc ARE3 transcriptional activity

Nrf2 has been identified as a target transcriptional factor essential for ARE transactivation. To determine if induction of ARE3-luciferase activity is linked to activation of Nrf2, we examined the effect of constitutively over expressing Nrf2 on ARE3 transcriptional activity. As detected by western blotting, Nrf2 nuclear levels were enhanced in Nrf2-overexpressing cells by 250% (data not shown). Transfection of an Nrf2 expression plasmid also led to robust induction of *Gclc* ARE3-driven luciferase activity (Fig. 2.3A). On the other hand, there was no induction of the embedded AP1 element over baseline, showing that Nrf2 neither directly nor indirectly trans-activates *Gclc* transcription through the AP1 component of ARE3 (Fig. 2.3A). The mutant ARE3 construct (*Gclc*ARE3 mut) was then co-transfected with the Nrf2 expression plasmid in H4IIE cells. Mutation of ARE3 abolished the Nrf2 response in reporter gene assays (Figure 2.3 B). These results indicate that the identified ARE3 is functional and mediates the activation of *Gclc* transcription by Nrf2.

Effect of Nrf2 siRNA on ARE3-mediated transcription of GCLC

We confirmed the role of Nrf2 cDNA overexpression in ARE3-mediated



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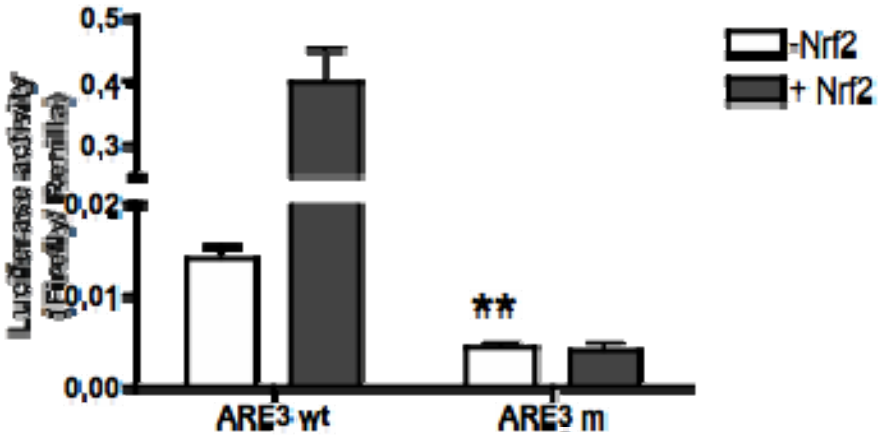

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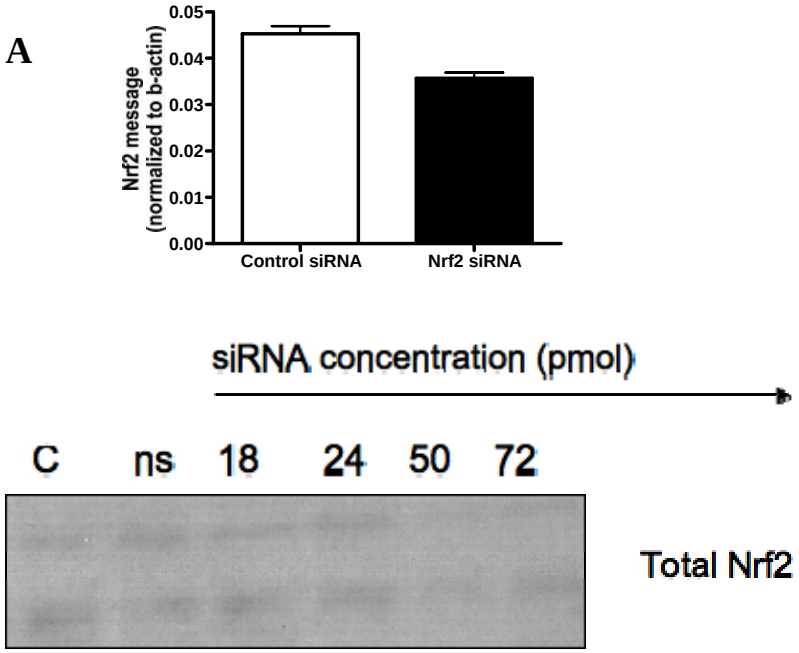
Figure 2.3 Nrf2 activates rat *Gclc* gene promoter activity through ARE3. (A) Nrf2 is important for ARE3-mediated *Gclc* promoter activity. H4IIE cells were transfected with 800 ng of pARE3-*rGclc*-Luc2, 600 ng of pcDNA3.1-Nrf2 (Nrf2) expression plasmid along with 20 ng of pHRL-CMV Renilla luciferase plasmid as a control for transfection efficiency. Nrf2 response of each reporter construct is indicated by fold of activity to control co-transfection with pcDNA3.1. Luc activity of the reporter construct alone was arbitrarily set as 100, and the mean values of relative luciferase activity from at least three independent experiments, each carried out in duplicate, are shown with the $\pm$ S.E. as detailed under the "Materials and Methods". *, p <0.01 relative to ARE3 alone (Tukey's post-hoc analysis). (C) Nrf2 does not activate ARE3 mutant. Nrf2 response of ARE3 mutant is indicated by fold of activity to control co-transfection with pcDNA3.1. The experiment was repeated three times, and similar

results were obtained. Mean difference is significant from control group at **p < 0.01 (Student's t test).

transcription of *GCLC* using small interfering RNAs (siRNAs) to inhibit the endogenous expression of Nrf2 in H4IIE cells. The transfection of H4IIE cells with a siRNA specific for Nrf2 reduced Nrf2 message and protein expression by about 30% (Fig. 2.4A). Nrf2 was detected as two bands of different molecular weights, which was confirmed by siRNA treatment against Nrf2. By contrast, the scrambled control siRNA did not affect expression of Nrf2 message or its protein levels as compared to endogenous levels in non-transfected cells (Fig. 2.4 A). It should be noted that the Nrf2 band appeared to be ~ 100 kD, although the expected molecular weight is 68 kD. The occurrence of the higher molecular mass of Nrf2 in Western blot analysis has been described by others and may represent either a Nrf2-actin complex (211) or be because of an abundance of acidic residues found in Nrf2 (212). Nrf2 siRNA was also co-transfected with *Gclc* ARE3 5'-luc2 or *Gclc*ARE3 (TRE) 5'-luc2, respectively, and its effect on *Gclc* promoters' activation was determined. Briefly, H4IIE cells were first transfected with Nrf2 siRNA. Four hours after the siRNA transfection, *Gclc* ARE3 or *Gclc* (TRE) ARE3 promoter-luciferase plasmids were transfected. Luciferase assays were performed 36 h after the second transfection. Cotransfecting the ARE–luciferase construct enabled the measurement of residual transcriptional ARE activity in Nrf2-depleted cells. The silencing of Nrf2 led to a significant abrogation in ARE3-driven luciferase activity (Fig. 2.4B). These data reveal that Nrf2 is directly involved in ARE3-mediated basal transcription of the rat *Gclc* gene.

Taken together, these studies indicate that the rat *GCLC* promoter contains one

functional ARE that is responsible for Nrf2-dependent basal transcription.



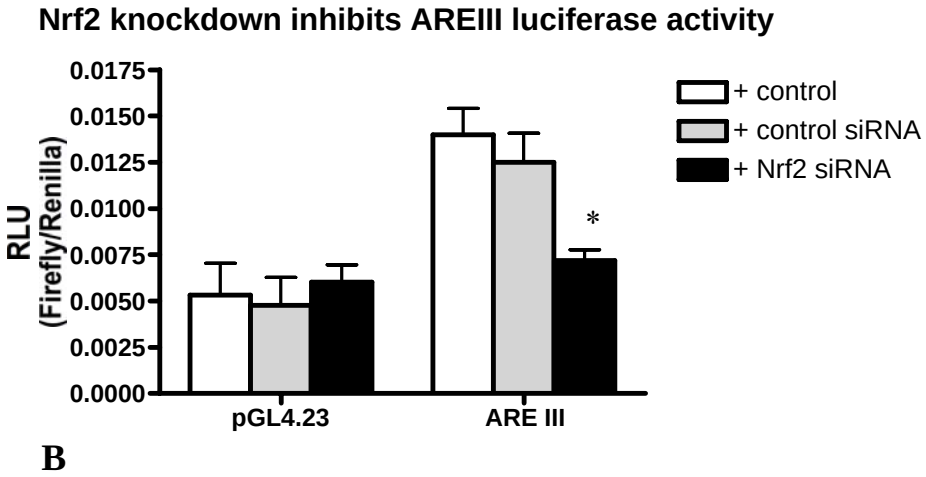


Figure 2.4 Effects of Nrf2 siRNA on ARE3-dependent *Gclc* transcription in H4IIE cells. H4IIE cells were transfected with 50 pM control siRNA or 50 pM Nrf2 siRNA as described under Materials and Methods, and then transfected with pARE3-*rGclc*-Luc2. (A) 24 hours after siRNA transfection, Nrf2 RNA levels and Nrf2 proteins in nucleus were detected by RT-PCR and Western blot, respectively. (B) Nrf2 knockdown inhibits luciferase activity of ARE3, but not the empty vector. *, $P < 0.05$ versus ARE3 control. Luciferase activities after treatment with control siRNA is shown as a negative control. Results are shown as mean $\pm$ S.E.M. of three independent

experiments.

2.5 Discussion

In the present study, we present evidence that rat *Gclc* is under the direct transcriptional control of Nrf2 on a basal level. This is supported by the following observations: (a) computer-based searches of the *Gclc* 5'-flanking region show three putative ARE elements at -2386 to –2377 exist in this gene; (b) the transfection experiments with *Gclc* ARE and AP1 promoter constructs indicated only one of the AREs (ARE3) and its embedded TRE element have transcriptional activity; (c) chromatin immunoprecipitation assays showed that Nrf2 binds only to ARE3 in the *Gclc* 5'-flanking region and (d) site-directed mutation of the ARE3 element abolishes luciferase activity. Collectively, the results of the present study establish that the transcriptional regulation of rat *Gclc* is governed by the Nrf2-ARE regulon. A schematic model of rat *Gclc* transcriptional regulation is depicted in Figure 2.5.

GSH is the most abundant non-protein thiol in cells and it plays key roles in multiple biological functions, including scavenging free radicals, conjugation and detoxification of electrophiles, and maintenance of normal cellular redox status. It has been well established that GSH levels are maintained via de novo synthesis through GCL gene expression. We demonstrate here for the first time that constitutive GCLC gene expression in the rat liver is regulated by an ARE (ARE3) present 3.8 kb upstream from the transcriptional start site. We also show that the transcription factor Nrf2 is directly involved in regulation of basal GCLC levels through ARE3.

Nrf2 binding to the ARE has been implicated as the master regulator of

constitutive regulation of *Gclc* at the genetic level in humans and mice. However, a

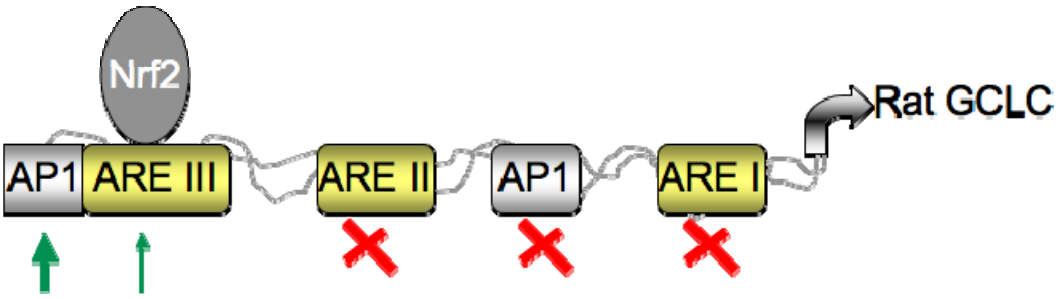


Figure 2.5 Schematic model of constitutive transcriptional regulation of rat *Gclc* gene. Out of the three AREs present in the *Gclc* 5'-promoter, only ARE3 exhibits basal transcriptional activity. ARE3 contains a 5'-flanking AP1 element which is responsible for enhancing most of its constitutive activity In Nrf2-mediated transcription, only the ARE3 is required for transactivation. Thus the Nrf2-ARE3 motif is a requirement for proper *Gclc* expression.

direct link for the role of Nrf2-ARE binding in basal *Gclc* gene regulation has never been demonstrated in rats. In deciphering the cis-regulatory element responsible for the basal transcriptional regulation of rat *Gclc*, there are differences of opinions and in some cases, even controversies (80, 81, 94, 133-135). Lu and co-workers reported that basal as well as inducible transcription of rat *Gclc* is regulated by an AP1 element located 450 bp upstream from the transcriptional start site (80). This study also did not identify any ARE in the rat *Gclc* promoter region. Subsequent studies including one from the same group reported that the Nrf2 is essential for rat *Gclc* transcription (133-135). In addition, it was reported that Nrf2 regulated the transcription of rat *Gclc* indirectly through the AP1 site (135). However, there are two significant caveats in these studies. First, it is important to recognize that this group analyzed only the first 2 kb upstream of the transcriptional start site, so the potential role of distal elements such as ARE3 was not assessed (80). Secondly, the rat *Gclc* promoter lacking the distal ARE elements was analyzed in fibroblasts of Nrf2 null mice, hence an accurate mechanism was not obtained (135). Nevertheless, the mechanism of transcriptional regulation of rat *Gclc* was an unsolved problem at the beginning of this study.

In previous reports investigating transcriptional regulation of rat *Gclc*, DNA binding assays were performed to assess transcription factors bound to consensus sequences since the complete sequence of the promoter had not been cloned. However, the DNA binding assay is limited by its inability to disclose the native state of competition between two reactive elements at one time. In this report, we obtained more direct evidence using chromatin immunoprecipitation, which involves

immunoprecipitating chromatin by Nrf2 and amplifying the TRE- or ARE-containing regions individually by PCR.

Several lines of evidence indicate that regulation of rat *Gclc* was linked to Nrf2 activation. For example, the ectopic expression of Nrf2 increased the luciferase activity of the ARE3 construct. Consistent with this notion, knock down of Nrf2 expression by siRNA effectively counteracted ARE3-dependent basal transcription of *Gclc*. Constitutive transcription of rat *Gclc* resulted directly from binding of Nrf2 to an ARE sequence on the *Gclc* gene that was located between -3901 and -3877 bases from the transcriptional start site. This result is consistent with other studies in human HepG2 cells that have shown the presence of Nrf2 in the *Gclc* ARE4 complex by electrophoretic mobility shift assay (79). The distal ARE/EpRE sites located in the human *Gclc* promoter region, between -3802 and -2752, including ARE3 and ARE4, are required both for constitutive expression and for induced expression of the human *Gclc* gene (117). Recently, it was reported that like other AREs, the human ARE4 in the *Gclc* promoter region consists of a consensus ARE sequence containing an embedded TRE element (130). The presence of a 5' AP1-like element is known to enhance NQO1 gene ARE-mediated gene expression (121, 213). However, the function of these regulatory sequences and the interaction between them has not been fully elucidated. Since Nrf2 binds only the ARE3 of rat *Gclc*, these data also suggest that it is ARE3 instead of other cis elements that is involved in constitutive *Gclc* regulation. The results here provide a molecular mechanism for transcriptional regulation of rat *Gclc* and provide the first direct evidence that Nrf2 targets rat *Gclc* as part of maintaining constitutive glutathione synthesis.

The AREs other than ARE3 showed more or less no Nrf2 binding or luciferase reporter activity because of unknown reasons. Subtle differences in the DNA sequence surrounding the core ARE site can modify the relative activity of the element (197). So far no universally applicable consensus sequence has been derived for the ARE (197, 214). However, it is noteworthy that the rat *Gclc* ARE3 responsive to Nrf2 contains a functional binding site for the AP1 family of transcription factors similar to the functional *Gclc* ARE4 in humans. The 5'-TGAC-3' tetranucleotide within the ARE3 core sequence resembles the half-site recognized by members of the AP-1 family (consensus: 5'-TGACTCA-3'). This also matches with results from Yang and co-workers where blocking AP-1 with dominant negative c-Jun lowered the basal expression of *Gclc* (198).

Another layer of complexity in that basal regulation of *Gclc* could be the result of cross-talk between ARE3 and its embedded AP1 site. Numerous studies have proposed a consensus ARE as 5'-RGTGACnnnGC-3' (where n = A, C, G, or T, R = A or G) after mutagenesis studies of the rat *GSTA2* and *NQO1* gene enhancers (124). Recently, it has been proposed that the sequence of the minimal ARE enhancer is (a/g)TGA(C/T/G)nnnGC(a/g) (215). After analyzing promoters of various ARE-containing genes, Wasserman & Fahl (125) suggested that the functional ARE is better represented by an extended consensus sequence 5'-TMAnnRTGAYnnnGCRwwww-3' (where W = A or T; M = A or C, 'core' consensus underlined). Furthermore, Erickson *et al (214)* suggested that the ARE consensus should be revised to 5'-RTKAYnnnGCR-3' (where K = G or T, Y=C or T) as a result of finding a functional ARE in the human

GCLM promoter region. Interestingly, a detailed mutagenesis study of the mouse *NQO1* ARE by Nioi *et al (197)* found that the G at position 14 (Wasserman numbering) was not essential for function of the enhancer, but the nucleotides marked 'n' at positions 4 and 12 were essential for function in mouse. This study suggested that a universally applicable ARE consensus sequence might not be possible (197).

Once translocated into the nucleus, Nrf2 forms heterodimers with other leucine zipper proteins; these dimers then bind the ARE and activate gene expression (205, 216). Nonetheless, the ARE-binding complexes are composed of variable mixtures of transcription factors. In addition to Nrf2, many other proteins, which may or may not be the dimerization partner of Nrf2, could also be activated and bind to the ARE, and thereby induce expression of rat *Gclc*. It has been demonstrated that many nucleoproteins can dimerize with Nrf2, such as small maf proteins and Jun family members (c-Jun, JunB, JunD) (145, 156, 157, 205). For example, under stimulated conditions, the transcription factors binding to a consensus human *Gclc* ARE complex included Nrf2, c-Jun, JunB, and JunD in HBE1 cells (134); in HepG2 cells, the proteins binding to a consensus *Gclc* ARE complex were composed of Nrf1/2, JunD, and small Maf proteins (79, 147). Further study on which nuclear proteins are involved in Nrf2 dimerization and rat *Gclc* activation is underway.

In this study, we found that basal transcription of rat *Gclc* occurs via the Nrf2/ARE3 complex since mutation of a previously uncharacterized ARE abrogates *Gclc* promoter activity. Moreover, ChIP assays detected increased Nrf2 binding to ARE3. Finally, a role for Nrf2 in the induction of *Gclc* promoter activity is also

demonstrated by the ability of Nrf2 siRNA to abolish the activation of *Gclc* promoter activity via ARE3.

Chapter 3

Characterization of A Rat Primary Hepatocyte Culture Model For Studying Age-Related Changes in ARE-Mediated Genes

Swapna V. Shenvi, Kate P. Shay, Brian M. Dixon, and Tory M. Hagen

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3.1 Abstract

It has been reported that the isolation and culture of primary hepatocytes can compromise cellular ability to constitutively express antioxidants and phase II enzyme genes, making it difficult to study their regulation ex vivo. The goal of the present study was to evaluate a collagen-based primary hepatocyte culture system to study age-related regulation of antioxidant response element (ARE)-mediated genes. Two million hepatocytes isolated from young (3 months) and old (24 months) rats were cultured on six-well plates pre-coated with Type I collagen. Williams E media fortified with fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, insulin, and dexamethasone was found to be an optimal support media for maintaining cell viability, but without significant cell proliferation. Viability studies using release of lactate dehydrogenase (LDH) activity as a means of quantifying membrane integrity suggested that hepatocytes taken from either young or old rats remained viable for at least 5 days in culture; however a significant induction in hepatocyte cell death was observed thereafter. Results show that steady-state tissue expression of key phase II detoxification genes [e.g. glutathione synthesizing genes and glutathione S-transferase 2A (GST2A)], which decline significantly in aging rat liver, also exhibited similar and extensive declines in cultured hepatocytes. Additionally, hepatocytes from both age groups retained their age-associated declines in terms of glutathione levels and NAD(P)H:quinone oxidoreductase 1 (NQO1) activity. Lastly, analysis of nuclear Nrf2 levels and Nrf2-ARE binding demonstrated that the aging differences in these parameters were maintained in cell culture. We conclude that the collagen-based cell culture system can be used to study ARE-mediated phase II gene regulation in primary hepatocytes from young and senescent animals, provided that experiments can be

conducted within a time frame of 5 days in culture. These data also support the hypothesis that aging compromises hepatocellular ability to maintain antioxidant gene status and upregulate Nrf2 nuclear levels.

3.2 Introduction

The liver is the chief organ involved in the detoxification and removal of xenobiotics. It plays a major role in conversion of toxic compounds to conjugated hydrophilic metabolites that can be readily excreted. The metabolism of chemicals usually involves two enzymatic stages commonly referred to as phase 1 and phase II detoxification (217, 218). Phase I detoxification is mostly carried out by cytochrome P450 (CYP) mixed function oxidases (217, 218), resulting in an oxidized metabolite that is further conjugated or reduced (phase II). Examples of phase II detoxification enzymes include those involved in glutathione (GSH) metabolism, [e.g. γ-glutamate cysteine ligase (GCL), glutathione S-transferases (GSTs)] and NAD(P)H: quinone oxidoreductase 1 (NQO1) (219-222).

The ability to detoxify a variety of toxins, mutagens, and xenobiotics is significantly impaired during aging (9). In particular, aged organisms have a reduced capacity to rapidly synthesize protective low molecular weight antioxidants, such as GSH and phase II detoxication enzymes (7, 9, 94, 223). Mechanisms responsible for these critical age-related deficiencies in the cellular stress response are unclear, but experimental evidence implicates a poor transcriptional regulation of redox-sensitive acute response (stress) genes (21). A case in point is the age-related decline in hepatic GSH levels. Recently, we showed that GSH levels decline with age because of lower amounts of its rate-limiting enzyme, GCL (94). This decline corresponded with lower nuclear levels of the transcription factor, NFE2-related factor 2 (Nrf2) and its attendant binding to the Antioxidant Response Element (ARE) present in the 5'-promoter of GSH-synthesizing genes (94).

Although these are important observations that appear to link the Nrf2-ARE system to a lesion in GSH synthetic capacity with age, the exact transcriptional mechanism governing these observations is currently unknown. Because of the nature of current experimental tools available to study transcription, it is difficult to answer this question in the intact liver or liver slices that have been used extensively in the past for detoxification studies (224). On the other hand, cancer cell lines are not a viable model for these studies as changes in GSH regulation during replicative senescence do not match liver GSH homeostasis (225, 226).

Freshly isolated hepatocytes isolated from young and old animals are a good model to study these age-related changes; however, they only survive for a few hours after the isolation process, if not placed in culture (12, 227). Thus, cultured hepatocytes hold many advantages over immortalized hepatoma lines or liver slices for aging research. For example, Suter and co-workers compared the expression of various phase 1 and phase 2 enzyme genes of liver tissue with liver slices, primary hepatocytes and two hepatoma cell lines, using microarray technology (224). Results from this study show that at any given time point, gene expression profiles of tissue slices matched most closely with the whole liver, while the two hepatoma cell lines had highly compromised levels of CYP450s and phase II genes such as metallothionein and GST2A. It is important to note that while primary hepatocytes differed in gene expression of most phase II enzymes from whole liver at 6 hours after plating, the changes in expression that culture induces were reversed to normal levels

after 24 hours in culture (224). Thus, cultured primary hepatocytes appear to be a suitable candidate to study age-related changes in the transcriptional regulation of phase II genes, provided that they are utilized for experiments after overnight culture.

Even though no studies, to our knowledge, have rigorously characterized cultured hepatocytes as to their suitability for examining age-related changes in detoxification capacity, a number of studies have shown that primary rat hepatocytes can be maintained in a functional state for at least a few days in culture (228-230). The duration of survival and prolonged expression of liver-specific functions *in vitro* are dependent upon culture conditions. In this regard, three groups of factors are influential: composition of the medium, extracellular matrix components, and cell-cell interactions (230, 231). The culture medium and constituent amino acids markedly govern hepatocyte performance *in vitro*. When long-term cultures of hepatocytes maintained on William's E medium were compared to similar cells maintained on Dulbecco's minimal essential medium, maximal albumin secretion occurred earlier with the former cells (232). William's E medium contains glutamate that potentiates hepatocyte response and modulates sensitivity to mitogens in primary culture (233). Moreover, an additive such as dexamethasone enhances the transcription of matrix proteins like collagen (232). Successful primary hepatocyte culture is also dependent on the addition of bovine serum to the medium. A likely explanation is that serum contains a bovine pancreatic trypsin inhibitor that helps to facilitate hepatocyte survival. This is because the plasma membrane of hepatocytes harbor a trypsin-like protease, which results in auto-degradation in serum-free culture (232).

In addition to composition of the medium, extracellular matrix is highly important for normal hepatocyte function. Suspensions of hepatocytes allowed to grow on glass or plastic surfaces without an extracelluar matrix establish polarity and form an epithelial monolayer that loses phase 1 and phase 2 gene expression rapidly (229). On the other hand, growth of hepatocytes on surfaces coated with collagen increases cell survival and maintains a high level of specific detoxication gene expression (234). Lastly, high cell density is essential for hepatocyte function and survival in long-term primary cell culture (231). This facilitates normal cell interactions and establishment of bile canaliculi that are essential for detoxification functions (235).

Several research groups have reported that liver-specific functions are compromised and that nuclear transcription of the antioxidant genes is significantly lowered following the isolation and culture of hepatocytes (228, 236-238). However, recent advances in primary cell culture environments have shown that liver-specific functions (e.g., CYP activity, GST expression) can be maintained at normal levels for up to 21 days in culture (238-241).

Despite many reports studying detoxification functions, no studies have been performed so far to study Nrf2-ARE-mediated transcriptional regulation in cultures of primary cells, regardless of the age of rats from which the hepatocytes are obtained. Furthermore, there have been very few studies to assess whether cells derived from young and old animals maintain their respective aging phenotype in primary culture

(242). Therefore, we have adopted a tissue culture approach to the problem by establishing a primary hepatocyte culture system that will permit us to address mechanistic questions related to age associated changes in cell function.

Using collagen-coated plates, the present study assessed the steady-state levels of certain phase II genes in cultures of hepatocytes isolated from young and old rats. These chosen genes have previously been documented to change during aging in the rat liver (111, 223, 243). Our results show that the aging phenotype is maintained for about 5 days in culture until a significant induction in hepatocyte cell death is observed. In addition, the age-related differences in the steady-state tissue expression of *Gcl* and *Gst2a* are maintained in culture. Hepatocytes from both age groups also retain their variations in terms of GSH levels and NQO1 activity. Lastly, analysis of nuclear Nrf2 levels and Nrf2-ARE binding demonstrate that the age-related decline in these parameters is maintained in cell culture.

3.3 Materials and Methods

Reagents

Williams E media was from Sigma Chemicals (St. Louis, MO, USA). Fetal Bovine Serum was from American Type Cell Culture. Collagenase D was from Boehringer Manheim. Histone H1 antibody from Calbiochem (EMD Biosciences, San Diego) was used as a means to verify equal loading in all lanes for immunoblot analysis. Nrf2 antibodies were obtained from Santa Cruz Biotechnology. All high performance liquid chromatography solvents were HPLC grade reagents from Fisher Scientific. All other chemicals were reagent grade or the highest quality available from Sigma.

Animals

Rats (Fischer 344, virgin male, outbred albino), both young (2–5 months: *n* 25) and old (24–28 months, *n* = 25; National Institute of Aging animal colonies), were acclimatized in the Oregon State University animal facilities for at least 1 week before experimentation. Animals were maintained on a standard chow diet, and food and water were given *ad libitum*.

Cell Isolation and Culture

Hepatocytes were isolated by a two step collagenase perfusion procedure as described previously (244). Following dissociation, cell viability was estimated by the trypan blue exclusion test and cells were used if viability was over 80%. Liver parenchymal cells were seeded at a density of 2 x 10^6 cells/ well of a 6-well plate. The

culture medium used was Williams E supplemented with 5% FBS, 2 mM L-glutamine, 5 IU/ml penicillin, 5 μg/ml streptomycin, 10 ng/L insulin and 1 μM dexamethasone. The cells were cultured at 37°C in an atmosphere of 5% CO2 and 95% air. Medium was renewed after 4 h to remove unattached cells and changed every day thereafter.

Cytotoxicity Assays

Samples were collected at various times during the culture process. Some samples were collected after a 4 hour attachment period in Williams E medium complete. The remaining samples were collected at 12, 24, 36, 48, 60, 72 hours post-plating. Cell death was determined by measuring the leakage of cellular lactate dehydrogenase (LDH) into the medium. LDH activity was measured both in media and cell extracts using a kit (Sigma). Before addition of the reagents, cell monolayers were lysed in 0.2% Triton X100 in water for 15 min at room temperature. Optical density was then measured at 340 nm.

GSH Analysis and Measurement of GCL activity

Hepatocyte cultures containing 2 x 10^6 cells were used to determine the amount of GSH. Briefly, cells were lysed in 10% perchloric acid (wt/vol) containing 5 mM EDTA. After deproteinization, hepatic GSH and glutathione disulfide (GSSG) concentrations were determined according to the method of Faris and Reed (245).

Measurement of nuclear Nrf2 levels

Nuclear extracts were prepared from liver tissue or hepatocytes by the method

of Dignam *et al*. (246). Protein (40 ug) was loaded in each well of a precast 12% Tris HCl polyacrylamide gel (Bio-Rad). Separated polypeptides were transferred to nitrocellulose membranes (Amersham Pharmacia) and probed with anti-Nrf2 antibodies at a 1:2,000 titer. Chemiluminescent detection was done by an ECL Western Blotting Detection kit from Amersham Pharmacia.

Electrophoretic mobility shift assay (EMSA)

Transcription factor binding to the ARE was determined by using an EMSA. Nuclear extracts were prepared as described earlier. All gel-shift assays were performed for three sample replicates in each group. A synthetic double-strand oligonucleotide probe for the ARE (5' -TGG GGA ACC TGT GCT *GA*G TCA CTG GAG-3') (Santa Cruz Biotechnology) was end-labeled by using [^{32}P] (Amersham Pharmacia) and T4 polynucleotide kinase (Promega). Binding reactions containing equal amounts of protein (9 ug) and labeled oligonucleotide probes were performed for 20 min at room temperature in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris, pH 8.0). Specific binding was confirmed by using 100-fold excess unlabeled ARE oligonucleotide as a specific competitor. Protein– DNA complexes were separated by gel electrophoresis by using 6% nondenaturing polyacrylamide gels followed by autoradiography for 18 h to detect the degree of retardation produced by binding to the probe.

Statistical Analysis

Data were expressed as mean $\pm$ SEM (standard error of the mean). Comparison

between young and old hepatocyte age groups was made by the Student's t-test for independent data. Differences were considered significant for p values < 0.05.

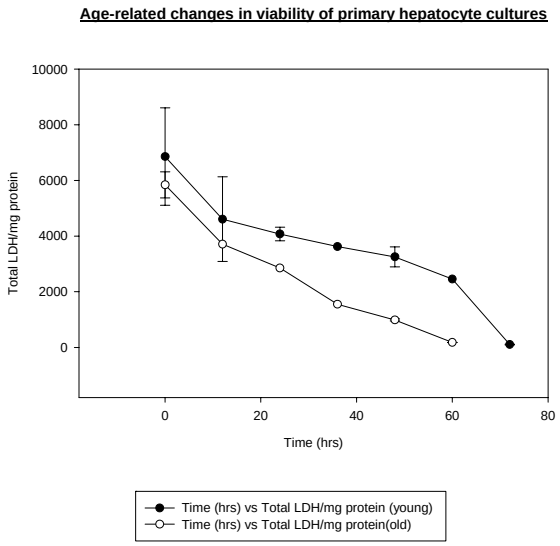
3.4 Results

Viability of hepatocytes at different stages in primary culture

The most commonly used cytotoxicity markers of *in vitro* toxicity tests in cultured hepatocytes detect changes which are the result of, or can lead to, cell death (e.g. enzyme leakage and dye uptake) (247). In the first set of experiments, we used lactate dehydrogenase (LDH) release as a marker of the appearance of membrane alterations of hepatocytes in culture over a 5-day period. Results presented in Figure 3.1A show that the viability of hepatocytes isolated from old rats is lower at all time points compared to those of young rats. This observation supports our hypothesis that cells collected from old rats lag behind their younger counterparts in adapting to cell culture conditions. We further analyzed whether old rat hepatocytes had a shorter survival time in primary culture. Figure 3.1B shows a representation of the LDH activity in hepatocytes from young and old rats as a function of their LDH activity at time zero in culture. These results suggest that old rat hepatocytes are no longer viable for experiementation after 60 hours in culture.

Age-Related Changes in GSH levels are maintained in primary hepatocyte culture

As GSH levels significantly decline in aging hepatic tissue and our objective of establishing a hepatocyte cell culture system is to study the mechanism of age-related GSH loss, we measured GSH in livers from young and old rats and compared the values obtained to that found in cultured cells. A comparison of tissue and hepatocellular GSH levels is shown in Figure 3.2A and 3.2B. Results show that GSH levels were significantly lower (50 + 6%) in liver tissue with age. GSH levels in



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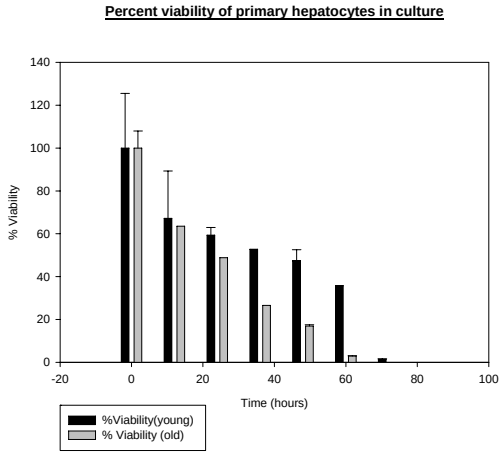
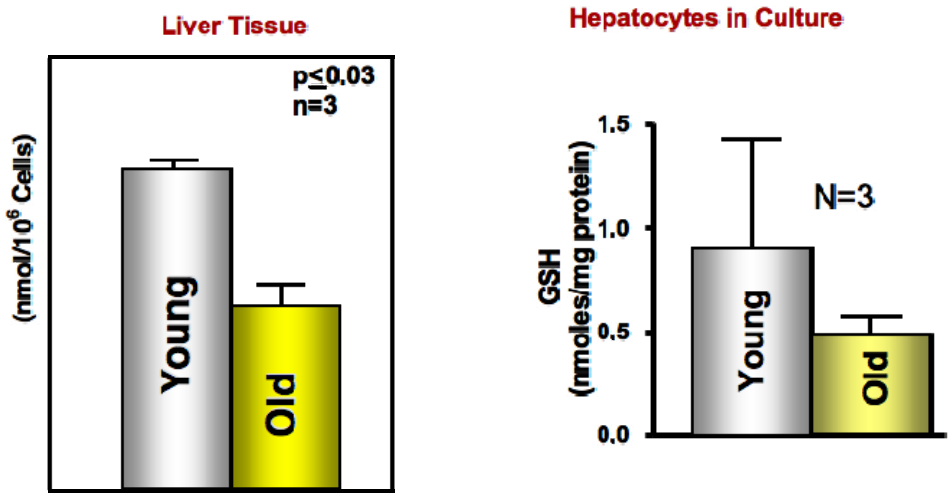


B

Figure 3.1 Viability of primary hepatocytes isolated from young and old rats at different stages in primary culture. (A) Age-related changes in viability of primary hepatocyte cultures as measured by the LDH assay. At indicated times, hepatocytes were scraped from plates, lysed and LDH activities measured. As a control, the LDH secreted in the medium was also measured (data not shown). The graph shows that heaptocytes from old rats are less viable at all times in culture, compared with hepatocytes from young rats. (B) Percent viability of primary hepatocytes in culture. Viability of hepatocyte cultures from young and old rats at zero time is indicated as 100. Results show that the rate of cell death in young and old rat hepatocytes is similar for up to 36 hours after plating. However, hepatocytes from old rats lose their viability more rapidly from the third day after plating, while hepatocytes from young rats maintian a more steady-state decline in viability. Results are representative of data from 5 young and 3 old rats.



A **B**

Figure 3.2 Primary hepatocytes in culture recapitulate the age-related loss of GSH. A) Liver tissue exhibits a 50 % age-associated decline in GSH levels. This loss of GSH is highly significant and has been demonstrated repeatedly. B) Hepatocytes from young and old rats were seeded on collagen-coated plates at a density of 2 million cells/well of a 6-well plate in Williams E medium supplemented with additives. The culture medium was replaced after 4 hours to remove dead cells. Hepatocytes were harvested in 10% PCA after 48 hours and the acid-soluble fraction was measured for GSH by HPLC. Results displayed in B show that hepatocytes from old rats in primary culture display a 45 % decline compared to those from young rats.

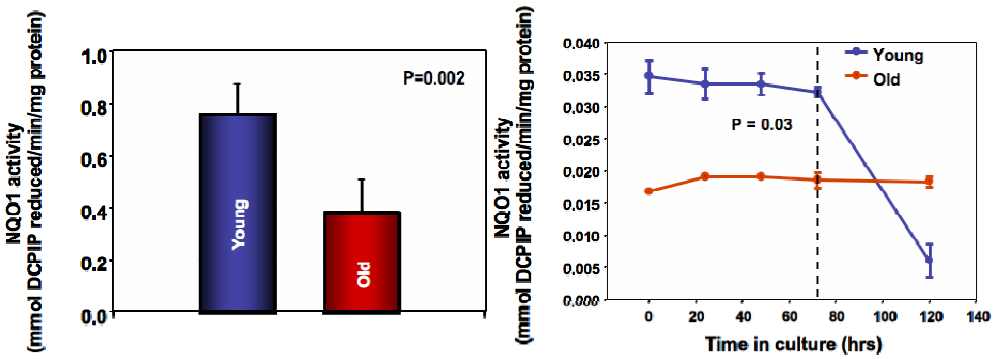
cultured hepatocytes taken from old rats exhibited a similar loss as that seen in tissue with age. Hence, the aging phenotype is maintained in terms of GSH levels in primary culture.

Age-Associated Differences in Phase II enzyme activity are retained in primary hepatocyte culture

We used NQO1 as a model gene to study phase II enzyme activity during aging in this primary culture model. NQO1 is an ideal enzyme marker for these studies as it is considered a quintessential ARE-regulated gene. Data from experiments measuring NQO1 activity shows that the loss with age is very similar in cultured hepatocytes compared to liver tissue (Figure 3.3A and B). Figure 3.3A shows that NQO1 activity in liver tissue is significantly down-regulated by of 62% ($P < 0.03$) with age. Measurement of NQO1 activity in cultured hepatocytes from young and old rats showed that NQO1 activity levels were similar to the in vivo activity (Figure 3.3B). Additionally, age-related differences in culture were maintained up to 60 hours after plating (Figure 3.3B).

Expression of ARE-Mediated Genes and their Aging Phenotype In Intact in primary hepatocyte culture

To further explore whether the culture model was appropriate to monitor age-related changes in phase II detoxification genes, the expression of the catalytic and modulatory subunit genes of GCL (GCLC and GCLM), the rate-limiting enzyme in GSH synthesis was examined. We have previously shown that expression of these



A. Tissue **B. Primary hepatocytes in culture**

Figure 3.3 Age-related loss of NQO1 enzyme activity is maintainted for up to 72 hours in culture. A) Liver tissue was young and old animals was homogenized and NQO1 activity measured via NAD(P)+ -mediated oxidation of 2,6-dichlorophenol indophenol (DCPIP). NQO1 was measured as the coumarin-inhibited activity of DCPIP oxidation. Results show that NQO1 activity declines by about 60% in the aging liver. B) Primary hepatocytes from young and old rats were placed in culture for 5 days and NQO1 activity measured over that period of time. Data demonstrate that NQO1 activity drops an order of magnitude when hepatocytes are placed in culture, however the age-associated phenotype with respect to NQO1 activity remains intact.

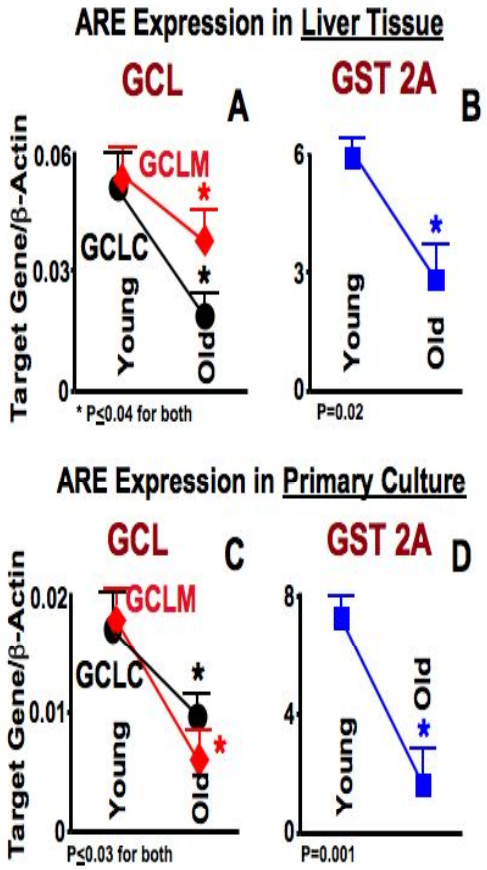
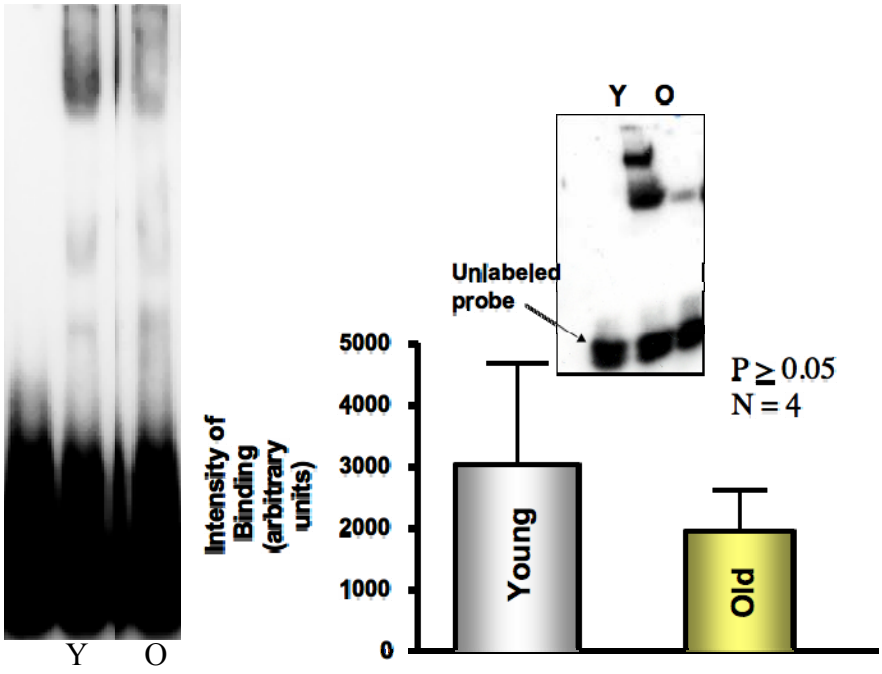


Figure 3.4 Expression of typical ARE genes decline with age both in vitro and in vivo. Using qPCR, expression of both *Gclc* and *Gclm* (A); and *Gst2a* (B) significantly decline in aged rat liver, indicating global loss of ARE-mediated gene transcription. Similar to the loss of ARE gene expression *in vivo*, expression of *Gclc*, *Gclm* (C) and *Gst2a* (D) decline by ≥ 50% in cultured hepatocytes from young and old rats.

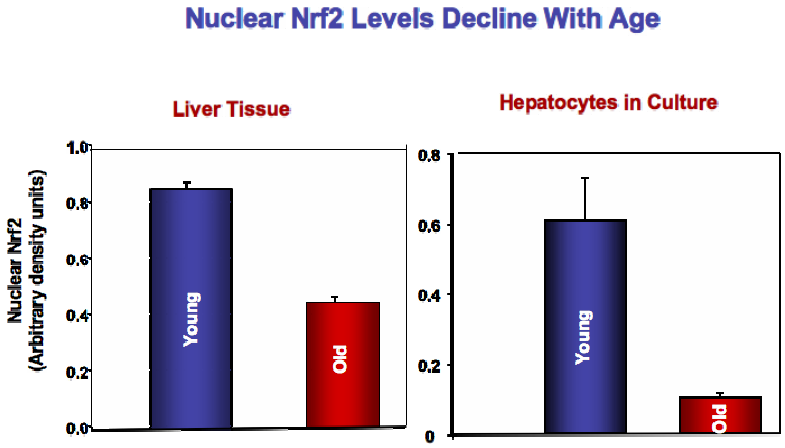
genes decline significantly in aging rat liver, which makes them appropriate for evaluating this culture system (94). A similar comparison was also done for GST2A, another phase 2 detoxification enzyme whose activity declines with age (248-250). Figure 3.4A and B show that *Gclc*, *Gclm* and *Gst2a* expression declines significantly with age in the liver tissue. Culture conditions induced a slight decline in *Gclc* and *Gclm* expression and a slight increase in *Gst2a* message levels, but the trends for an age-related loss in all these phase 2 detoxification genes was similar to that observed for intact liver tissue (Figure 3.4 C & D).

Downregulation of transcription factor complex at the ARE and nuclear Nrf2 levels in aging

As the expression of ARE-mediated genes declined in the aging rat liver, we investigated whether lower transcription factor binding to the ARE was responsible for this loss. Electromobility shift experiments (EMSA) with a consensus ARE probe shows that there is less overall transcription factor binding to this sequence in aged liver tissue and also in cultured hepatocytes from old rats, when compared to their respective young controls (Figure 3.5A and B). Because Nrf2 is the main transcription factor that to the ARE, we measured the amount of Nrf2 available in the nucleus for transcriptional activity by western blot. Results show that nuclear Nrf2 decreases by approximately 40% with age in the liver tissue (Figure 3.5C); however this age-specific loss is more pronounced in cultured cells (Figure 3.5 D). This suggests that hepatocytes from old rats are unable to upregulate Nrf2 under stress in culture conditions, showing that the transcriptional mechanism of ARE-mediated



A **B**



C **D**

Figure 3.5 Hepatocytes in culture mimic the in vivo phenotype in terms of nuclear Nrf2 levels and transcription factor binding to the ARE. (A & B) Transcription factor binding to a consensus ARE element was detected by EMSA using a synthetic oligonucleotide probe. Complementary DNA fragments corresponding to the ARE were labeled with biotin at the 5'-end, annealed and used as probe to measure age-related changes in the binding of transcription factors in vitro. Binding reactions were performed for 20 minutes with nuclear extracts from young and old rat liver tissue (A) or primary hepatocytes of young and old rats (B). Binding of transcription factors to probe is indicated by a retardation of the probe on a native

gel and was detected by streptavidin. Results show an age-specific decline in transcription factor binding to the ARE in liver tissue as well as primary hepatocytes in culture. (C & D) Steady-state levels of nuclear Nrf2 decrease with age. Nuclei were isolated from liver tissue (C) or primary hepatocytes in culture (D) and Nrf2 levels detected by western blot. Data presented above suggests that tissue nuclear Nrf2 declines to the extent of 45 % with age, however, there is a more pronounced age-related loss of Nrf2 when hepatocytes are placed in primary culture.

stress response is impaired with age.

3.5 Discussion

The main findings of the present study show that hepatocytes from young and old rats, cultured using collagen as a support, i) adopt a differentiated phenotype, and (ii) maintain the ability to constitutively express phase 2 enzyme activity and gene expression for at least 5 days in culture. These observations indicate that cells retained the ability to respond to the rigors of collection and culture. These results are also in contrast to previously published data reporting that primary hepatocyte cultures rapidly lose the ability to express antioxidant genes ex vivo (237, 251-254).

This discrepancy in data can be explained by looking at the time of measurement of these antioxidant genes in culture. Most of the previous studies (251-253) conducted gene expression studies in the first 24 hours of culture as opposed to this study. Primary cultured hepatocytes have been shown to undergo a "nonresponsive' period until 24 hours after plating, during which time phase 2 enzyme inducers fail to upregulate enzyme activity (230, 255). It is therefore possible that altered expression in cultured hepatocytes at early time points after plating (i.e. less than 24 hr) was masked either by the rapidly declining levels of phase II enzymes or by a general lack of cellular responsiveness during these early time periods. Baker *et al* recently studied expression of various phase 1, phase 2, and antioxidant genes in primary rat hepatocyte cell culture using an Affymetrix microarray (256). Results from their microarray analysis show that expression of antioxidant genes like hemeoxygenase1, metallothionein, and heat shock proteins rapidly decline in the first

four hours after plating, but start to recover after 24 hours, such that they are not significantly different from tissue levels (255). Thus, it is necessary to have a sufficient incubation time after plating to allow phase 2 genes to return to their basal levels of expression.

At the beginning of this study, we postulated that cells collected from aged rats would lag behind their younger counterparts in adapting to culture conditions. Our results show that hepatocytes isolated from young and old rats maintain their respective aging phenotype compared to normal liver tissue. Additionally, they also suggest that hepatocytes collected from old rats may be less competent than cells isolated from younger animals at withstanding the environmental insults associated with disruption of the liver and long-term culture. This is especially demonstrated by the extremely low LDH activity of old rat hepatocytes in primary culture for 5 days. Conversely, Nrf2, the main transcription factor regulating ARE-mediated stress response is not maintained to the same levels in old rat hepatocytes compared to age-matched liver tissue.

It has been shown previously that the regulation of some liver-specific genes can be significantly altered when hepatocytes are placed in culture; transcriptional regulation is lost or greatly decreased, resulting in the predominance of post-transcriptional regulation (257-263). But the addition of dexamethasone can restore the transcriptional regulation of these genes (258, 261-263). The results from this study indicate that the hepatocyte primary cell culture system that we have developed reflects, relatively accurately, the most important features of age-related changes in

ARE-mediated gene regulation in cell culture and should, therefore, be of immediate use in the study of the regulation of these genes during the aging process. At the same time, minor differences do exist between this cell culture system and liver in the transcriptional regulation of phase II genes, especially, in terms of greater age-related loss of nuclear Nrf2 levels, but further development should improve the usefulness of this cell culture system for the study of ARE-mediated gene regulation as well as for the study of other liver-specific genes.

In summary, the main findings of the present investigation establish that primary hepatocytes isolated from young and senescent animals and cultured with collagen adopt a differentiated phenotype and maintain the ability to express phase II enzyme genes. Although our results suggest that collagen may provide primary hepatocytes with an environment that promotes cellular organization closer to that observed in vivo, changes in phase 2 enzyme activities and LDH activity were evident at time points after 4 days ex vivo. Accordingly, we can conclude that the the collagen system minimizes, but does not eliminate, environmental factors that lead to altered gene expression in cultured cells. However, it is important to note that all the parameters for ARE-regulated genes examined as well as Nrf2 levels and ARE activity were maintained at or near control levels for the first 5 days ex vivo, suggesting that an adequate window of time exists for interventional studies using this preparation. With this outcome in mind, we find that the present data show that cells derived from young animals maintained LDH activity as well as ARE-mediated enzyme activity at or near control levels longer than cells derived from old rats. These

results are consistent with the hypothesis that age-related deficiencies in ARE-mediated transcriptional regulation of phase II genes contribute to declining stress tolerance in the elderly population.

Chapter 4

Age-Related Alterations in Nrf2-Mediated Transcriptional Control of Glutathione Synthesis

Swapna V. Shenvi and Tory M. Hagen

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4.1 Abstract

The aging rat liver is characterized by a decreased detoxification capacity, which is exemplified by the age-related decline in phase II-mediated detoxication. In particular, glutathione (GSH) levels decline with age, which, in turn, stems from a steady-state loss in nuclear levels of NFE2-related factor 2 (Nrf2), the controlling factor for the expression of GSH-synthesizing genes. We used the catalytic subunit of γ-glutamate cysteine ligase (GCLC) as a model to study the precise mechanism how age-related loss of Nrf2 induces transcriptional changes in the antioxidant response element (ARE) transcriptional complex. Results from chromatin immunoprecipitation experiment show that Nrf2 is enriched in lower amounts on the active "ARE3" promoter located -3.9 kb relative to the transcription start site in aged rats. We also present evidence showing that the active ARE3 transcriptional complex is converted to a repressive motif during aging as shown by binding of the repressor, Bach1, and lower ARE3-driven luciferase activity. Furthermore, Nrf2 occupies an alternate ARE site (-2.2 kb; "ARE-like") in livers of old rats, indicating an age-specific promoter switch. Our results show that the conversion of Nrf2 binding from an active ARE element to this ARE-like element is not adequate to maintain basal expression of hepatic GCLC in old rats, which provides a possible mechanism for the age-related loss of glutathione synthetic and other phase II enzymes.

4.2 Introduction

Aging is a multifactorial process leading to a multitude of physiological changes, often detrimental, that leads to elevated risk for morbidity and mortality. A constant hallmark of aging throughout mammalian life is a decreased response to both exogenous and endogenous stresses (21). In the liver especially, this inadequate stress response is reflected by a diminished detoxification capacity (34, 225, 264). This is particularly disadvantageous in the elderly in terms of adverse drug interactions. It appears that antioxidant defenses do not keep pace with the oxidants encountered in aging (8, 9, 12, 265, 266). In fact, the 'free radical theory of aging' proposed by Harman in 1954 postulates that the aging phenotype is characterized by an increase in pro-oxidant state and a decline in antioxidant levels (7, 267). The insufficiency of antioxidant defenses in aging is exemplified by lower levels of certain low molecular weight antioxidants like vitamin C and glutathione (GSH). We and others have previously shown that GSH levels decline in the aging liver of Fischer 344 rats (94, 132, 242).

The de novo synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and subsequent formation of GSH from γ-glutamylcysteine and glycine (73, 190). Glutamate cysteine ligase (GCL) catalyzes the first and rate-limiting step of *de novo* synthesis, while the second step is by glutathione synthetase (GS) (73, 190, 200). Hence, GCL is a major determinant of the overall capacity of GSH synthesis. Data from many laboratories have conclusively shown that hepatic GCL activity, protein levels and gene expression are significantly lower in aging rats (94, 113, 134).

GCL protein is a heterodimer that can be dissociated under non-denaturing conditions into a catalytic (GCLC, 73 kDa) and a modulatory (GCLM, 29 kDa) subunit, which are encoded by separate genes (268). Although the heavy subunit contains the entire catalytic activity, association with the regulatory GCLM present in far less amounts than GCLC can modulate its activity (192). The enzyme can be regulated at kinetic, post-translational and transcriptional levels (50, 51, 192). However, the regulation of GCL at the transcription level produces a more persistent effect and thus is more important for the maintenance of GSH homeostasis in response to oxidative stress (269, 270).

Recently, we showed that the age-related loss of GCL is linked to lower nuclear steady-state levels of the transcription factor, NFE2-related factor 2 (Nrf2) (94). Coincident with lower nuclear Nrf2 levels is less binding of Nrf2 to its cognate DNA-binding region in the 5'-flanking region of GCL genes, the antioxidant response element (ARE). Consequently, lower basal GCL expression and GSH levels are maintained in the aging rat liver. Though a lesion in Nrf2 status in the nucleus has been implicated as the culprit of age-associated decline in GSH, the exact consequences of Nrf2 loss on the GCL transcriptome still remains to be elucidated.

We used GCLC as a representative GSH-synthetic gene to study the transcriptional mechanism of age-related insufficiency in GSH synthesis. We previously showed that the 5'-flanking region of the rat GCLC gene has three AREs, but only one of these sequences (ARE3) displays Nrf2-binding and transcriptional activity. Therefore, we hypothesized that in aged rats, there is lower Nrf2 binding to

the *Gclc* ARE3, leading to a transcriptional remodeling, which contributes to a decline in its expression.

In this paper, we show that, hepatocytes from young and old rats placed in primary culture display an an age-related decline in steady-state Nrf2 nuclear levels. Using chromatin immunoprecipitation (ChIP) analysis, we show that less Nrf2 is recruited to the ARE3 promoter with age and that the active ARE3 transcriptional complex in the young rat liver is replaced by a partially inactive complex in livers from old rats. This repressive motif is characterized by the presence of the Nrf2 repressor, Bach1, and an absence of the co-activator CREB-Binding Protein (CBP). Additionally, aging induces a weak enrichment of Nrf2 to a downstream "ARE-like" promoter (-2.2 kb), but this promoter switching is not adequate to maintain GCLC levels. Our data provide a molecular basis for the observed age-associated loss of GCL levels; the distribution of Nrf2 among two ARE promoters and the formation of a repressive complex at the ARE3 leads to a failure of old livers to maintain GSH levels during aging.

4.3 Materials and Methods

Chemicals and antibodies

Restriction enzymes and T4 DNA ligase for subcloning were from New England Biolabs (Boston, MA). The dual-luciferase reporter assay system and reporter plasmids, pGL4 minimal promoter vector and phRL-CMV vector were from Promega (Madison ,WI). Expression vector for Nrf2 (pcDNA3.1-Nrf2) was a kind gift provided by Dr. Jaiswal at Baylor College of Medicine, Houston, Texas (205). Custom oligonucleotides used in PCR cloning, subcloning, and DNA sequencing were purchased from Invitrogen (Carlsbad, CA). Sequence service was provided by CGRB at OSU. Rabbit anti-Nrf2 (H-300), anti-lamin B1, small maf antibodies and Nrf2 siRNA and scrambled sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other antibodies were purchased from the following suppliers: c-Jun and Bach1 (Abcam), c-Fos (eBioscience), CBP (Upstate Biotechnology, New York), and IgG (Calbiochem). Protein A-Sepharose was purchased from Sigma. All chemicals used were at least analytical grade.

Animals

Rats (Fischer 344, virgin male, outbred albino), both young (2–5 months: n =12) and old (24–28 months, n=25; National Institute of Aging animal colonies), were acclimatized in the Oregon State University animal facilities for at least 1 week before experimentation. Animals were maintained on a standard chow diet, and food and water were given ad libitum.

Hepatocyte Isolation and Culture

Rats were anesthetized using diethyl ether (Fisher Sci., Fair Lawn, NJ) and the liver was exposed by a midlateral incision in the abdomen. Rats were sacrificed by cutting through the diaphragm and severing the vena cava. The animals were consistently sacrificed between 9:00 to 11:00 AM to minimize differences in food intake and diurnal variability. Liver tissue from young (3–5 months) and old (20–24 months) rats were dispersed into single cells by collagenase perfusion (Moldeus ***et al.***, 1978). Cell number was assessed by using a hemocytometer, and viability was determined by trypan blue [0.2% (wt/vol) in phosphate-buffered saline (PBS)] exclusion. Viability was normally greater than 90% in both age groups.

Freshly isolated hepatocytes were plated on collagen-coated culture dishes in William's Medium E supplemented with 5% FBS, 1 mM dexamethasone, 100 ng/ml insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin for 4 h in 5% CO2 at 37°C to allow attachment to the dishes. The medium was then replaced with fresh supplemented William's Medium E, and the cells were cultured for an additional 48 h before chromatin immunoprecipitation or transfection with the appropriate ARE-luciferase constructs.

Preparation of nuclear extracts and Nrf2 analysis

Cells were scraped into ice-cold lysis buffer (10 m Hepes, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 10 mg/mL pepstatin A, 0.5 mM DTT, and 0.4% NP-40), incubated for 10 min, and centrifuged at 14,000 g for 3 min at 4 °C. The resulting nuclear pellet was resuspended in extraction buffer (20 mM HEPES (pH 8.0), 0.4 M NaCl, 1.0 mM EDTA, 1 mM DTT, 0.5 mM

PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 10 mg/mL pepstatin A, and 10% glycerol), kept on ice for 15 min, and centrifuged at 14,000 g for 5 min at 4 °C. The supernatant containing the nuclear proteins was resolved by SDS-PAGE and Nrf2 levels determined by western blotting using Nrf2 antibody (1:1000) as described before.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation analysis was conducted using control rabbit IgG, anti-Nrf2, anti-small maf, anti-c-Jun, anti-c-Fos, anti-CBP, and anti-Bach1 antibodies. PCR primers are described in Table 4.1. Eighteen million hepatocytes were used for each chromatin immunoprecipitation. Briefly, proteins and DNA were cross-linked with 1% formaldehyde for 10 minutes at room temperature and cells lysed in SDS-lysis buffer containing protease inhibitors and then sheared to an average length of 500-800 bp by sonication using a Sonic Dismembrator Model F60 (Fisher Scientific, Pittsburgh, PA). Sheared chromatin was immunocleared with salmon sperm DNA/protein A-sepharose and 10% of the precleared chromatin was stored and labeled as "input DNA". The remaining chromatin was immunoprecipated with IgG (control) or Nrf2, small maf, c-Jun, c-Fos, CBP or Bach1 antibodies (2 μg) overnight at 4°C. Immune complexes were adsorbed onto salmon sperm DNA/protein A-sepharose beads. Immunoprecipitates were washed sequentially with wash buffers to reduce background. Protein–DNA complexes were eluted from the antibody with elution buffer (1% SDS, 0.1 M NaHCO3) and formaldehyde cross-links reversed by addition of NaCl (5 M) and heating at 65 °C overnight. DNA was purified using the

QIAquick purification kit (Qiagen, Valencia, CA) and PCR performed using primer pairs that spanned each of the ARE and AP1 elements. PCR products were quantified using SYBR Green Mastermix (New England Biolabs) with the Mini Opticon 2 Real-Time PCR Detection System (Bio-Rad). Specific enrichment of DNA by anti-Nrf2 antibody was calculated by subtracting the PCR value of normal IgG from that of anti-Nrf2 antibody and by normalizing that value to the PCR input. Triplicate PCR reactions were conducted for each sample, and the experiments were repeated at least thrice. The specificity of the PCR products was confirmed by melting curve analysis and size (agarose gel electrophoresis).

Construction of luciferase reporter vectors

The ARE- and ARE-like-luciferase reporter plasmids were generated using the pGL4-minimal promoter vector (Promega) containing a minimal TATA promoter upstream of the firefly luciferase gene. The sequences of the inserts used in the different plasmids are summarized in Table 2.1. Single-stranded oligonucleotides were first annealed to form double-stranded oligonucleotides and then ligated into the pGL4.23[minP] vector following the manufacturer's instruction (Promega). Each of the vectors was engineered by inserting 3 copies of each of the ARE elements present in the rat *Gclc* 5'-flanking region. The three different *Gclc* 25-bp ARE (3X)-driven luciferase reporter constructs [i.e., *pGL4.23Gclc*-3xARE1-Luc2, *pGL4.23Gclc*-3xARE2-Luc2, and *pGL4.Gclc*-3xARE3Luc2] and the *Gclc* "ARE-like" (3X)-driven luciferase reporter construct [Pgl4.23*Gclc*-3XARE-like-Luc2] were made by insertion of the appropriately hybridized complementary oligonucleotides [75 bp] with 2-bp

overhangs into the *Xho*I/*HindIII* restriction sites of the pGL4-minimal promoter reporter vector. TOP10 competent cells were transformed with the recombinant DNA after ligation for amplification. After the plasmids were generated, the DNA sequence of the inserts was verified.

Hepatocyte transfection and luciferase assays

Reporter gene assays were used to determine the transcriptional activities of individual *Gclc* ARE elements and the ARE-like element in primary hepatocyte cultures from young and old rats. Transient transfections were done in hepatocytes cultured on 6-well collagen-coated plates for at least 48 hours using the Effectene Transfection reagent (Qiagen, Valencia, CA). The cells were transfected with 1.6 μg of *Gclc*-luciferase plasmids. Briefly, the DNA and 13 μl of Enhancer were dissolved in buffer EC from the kit to a total volume of 100 μl. The DNA–enhancer mixture was incubated at room temperature for 5 min. After incubation, 5 μl of Effectene transfection reagent was added to the mixture, mixed, and incubated at room temperature for 10 min to allow transfection–complex formation. Medium (1 ml) was added to the mixture and mixed. The mixture was then immediately added to the well containing the cells and 1 ml of fresh medium. The total amount of plasmid DNA for transfection was adjusted by empty expression vector (pGL4.23). 0.02 μg of the control plasmid phRL-CMV encoding *Renilla* luciferase was included in each transfection to account for variability in transfection efficiency. In some cases, 0.5 μg of Nrf2 expression plasmid (pcDNA2.1 Nrf2) was co-transfected with luciferase reporter constructs. Thirty-six hours after transfection, cells were harvested with 1 x

passive lysis buffer (Promega), and the supernatant was collected by brief centrifugation. Transcription activity was determined by the expression of firefly luciferase and was normalized to the renilla luciferase levels by using a dual luciferase reporter assay kit (Promega) on a Biolumat LB9505 luminometer (Berthold Detection Systems, Pfhorzeim, Germany). The means of at least three independent experiments, each carried out in duplicate, are shown with the ± S.E.

Statistical Analysis

The data are expressed as the means ± S.E. Statistical analysis was performed with the GraphPad Prism software version 3.03 (GraphPad Software Inc., San Diego, CA). We used a two-tailed Student's *t* test to compare the luciferase activity of individual *Gclc* promoter constructs. A *P* value less than 0.05 was considered to be significant. One-way analysis of variance (ANOVA) was used when multiple comparisons were made, followed by Tukey's post-hoc analysis for multiple comparisons to a control.

Table 4.1 Primers used for ChIP analysis of *Gclc* Promoter Elements

Gclc 5'-Element	Primers used for ChIP analysis
ARE1	FP: 5'-TTGCAAAACATTAATCGAACAACTA-3' RP: 5'-TTACTGGTTATTTACATGCGTTCAT-3'
ARE2	FP: 5'-TTCCTCCTGACAGTGAGTTGACACTCTGGA-3' RP: 5'-TTTTTAAGGCAGCTACTGAAAAGTC-3'
ARE3	FP: 5'-AAGAAGCCAAGAAGGAAGTGTG-3' RP: 5'-ACGAGAAGAAGCTCTGGGTCTT-3'
"ARE-like"	FP: 5'-CCTGTTTTTACATACCAAAACAGACC-3' RP: 5'-CTGAGCCAATTCAAACAGATTAGAT-3'

Note: 'FP' and 'RP' denote forward and reverse primer, respectively.

4.4 Results

Nuclear steady-state levels of Nrf2 decline with age

Nuclear Nrf2 levels were measured to establish the extent of age-related changes in this key transcription factor. Compared with hepatocytes from young rats, those from old animals exhibited a significant 83% decline ($P < 0.05$) in steady-state nuclear Nrf2 levels (Figure 4.1 A). To ascertain whether nuclear Nrf2 reflected changes in the cytosolic compartment, cytosolic cell fractions were obtained by differential centrifugation and Nrf2 levels determined by western blot. Results show that there is a non-significant age-associated change in cytosolic Nrf2 compared to the nuclear fraction (Figure 4.1 B). These results show that Nrf2-mediated transcription in aging is primarily governed by its nuclear translocation and retention.

Nrf2 is enriched to a lower level at Gclc ARE3 in hepatocytes from old rats

Since GCLC is the rate-limiting enzyme in GSH synthesis, we used it as a model to study the transcriptional consequences of lower nuclear Nrf2 levels in aging and whether it contributed to the age-related loss of GSH. There are multiple ARE sequences in the *Gclc* promoter and it is not known whether aging affects Nrf2-ARE binding universally or to certain ARE promoters. To determine the consequences of lower nuclear Nrf2 levels at individual ARE promoters, we performed chromatin immunoprecipitation (ChIP) assays using an Nrf2 antibody. Figure 4.2A shows a schematic representation of the three ARE elements and an "ARE-like" element in the

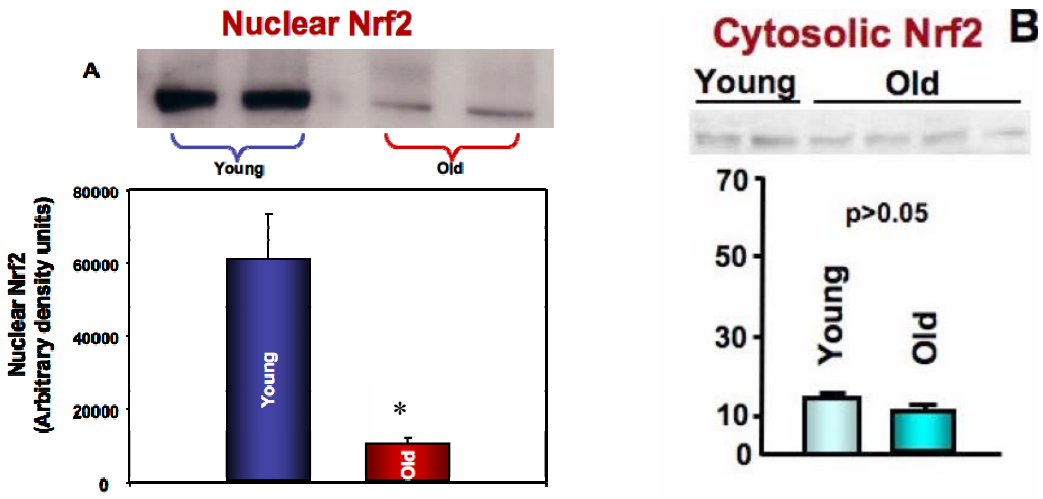


Figure 4.1 Nuclear levels of Nrf2 preferentially decline with age. Hepatocellular nuclear and cytosolic fractions were isolated and subjected to Western blot analysis for Nrf2 levels. Resulsts show that nuclear Nrf2 levels decline by > 50% ($P < 0.05$) with age (A), while cytosolic Nrf2 levels (B) diminish to a lesser degree. N=3 (young) and N=4 (old).

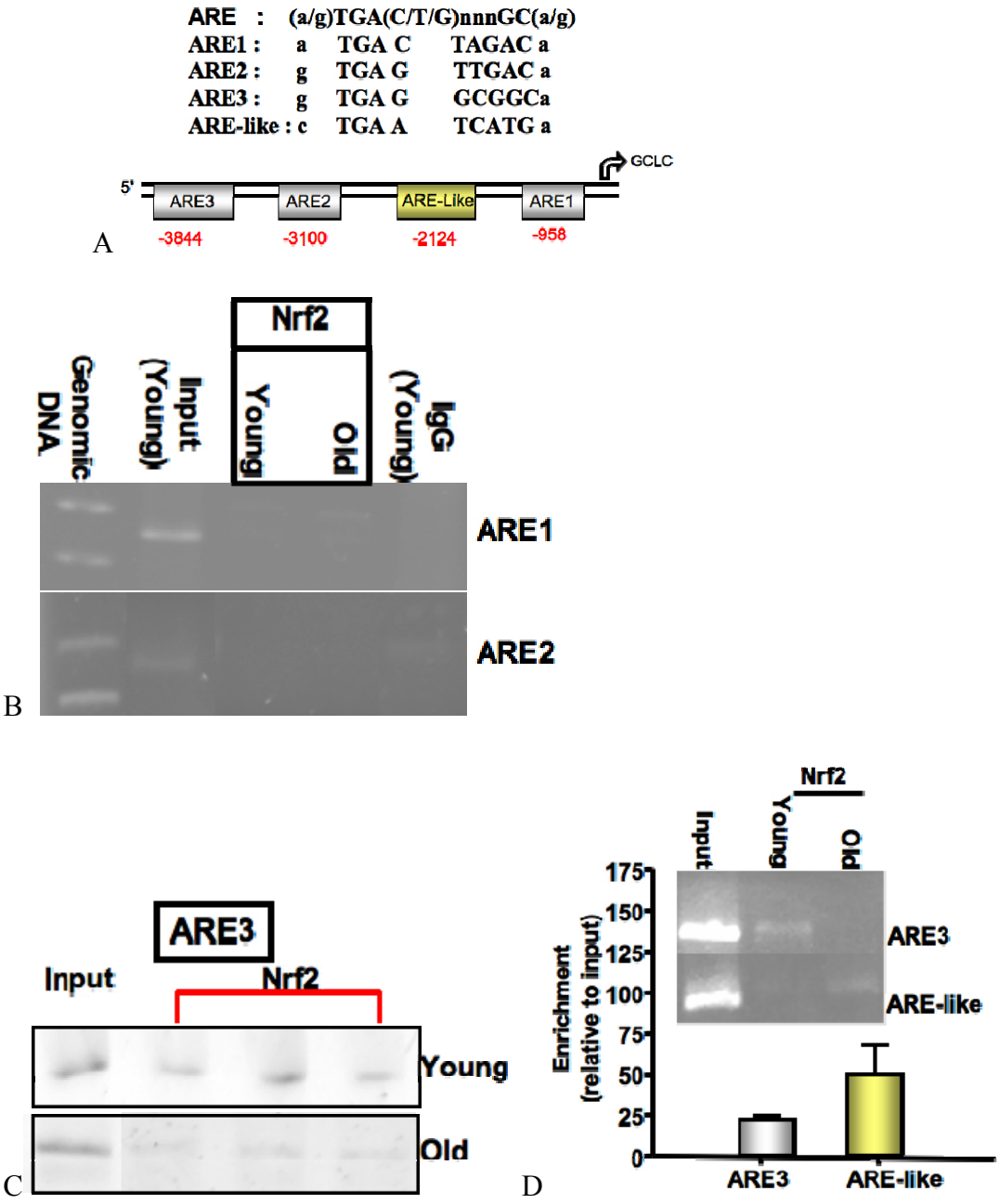


Figure 4.2 Aging induces promoter shifting of Nrf2 from ARE3 to an alternate ARE site. (A) Schematic representation of 5'-flanking region of *Gclc* showing locations of various AREs and the ARE-like element. Also depicted are the sequences of all the *Gclc* ARE elements and the ARE-like site compared to the consensus ARE. Note that the ARE3 sequence corresponds most closely to the ARE consensus. (B) Nrf2 does not bind to the ARE1 or ARE2 elements in hepatocytes from both young and old rats, showing that these two sites are not involved in the transcriptional regulation of *Gclc*. Genomic DNA and sheared input chromatin are shown as positive controls while the DNA amplified by IgG immunoprecipitation is shown as negative control. (C) Amount of Nrf2 binding to *Gclc* ARE3 declines with age. ChIP assays

were performed on young and old rat hepatocytes using an antibody to Nrf2 and amplifying the region spanning the ARE3 using specific primers. The sheared input chromatin is used as a positive control. (D) Nrf2 binds to the ARE-like promoter only in hepatocytes from old rats. Chromatin immunoprecipitation shows that Nrf2 is more enriched at the ARE-like promoter (50 ± 3; $P<0.05$) compared to the ARE3 promoter with age. No Nrf2 was detected at the ARE-like promoter in young rat hepatocytes, showing that the promoter switching was age-specific. Results are representative of ChIPs performed on hepatocytes from 3 young and 3 old animals.

5'-flanking region of rat *Gclc*. Results show that Nrf2 does not bind to either ARE1 or ARE2 in either hepatocytes from young or old rats (Figure 4.2B). However, Nrf2

associates with the ARE3 promoter in hepatocytes from young rats and this binding decreases significantly (60 $\pm$ 9 %, P <0.05) in old rat hepatocytes (Figure 4.2B & 4.2C). Thus the loss of Nrf2-ARE3 binding in old rat hepatocytes is consistent with lower nuclear Nrf2 levels.

We further asked the question whether the "ARE-like" element displays any Nrf2-binding activity. ChIP analysis showed no detectable binding of Nrf2 to the "ARE-like promoter" in young rat hepatocytes, indicating that Nrf2 association with the ARE sequence (ARE3) at -3901 bp was solely responsible for promoting *Gclc* expression. In contrast, Nrf2 was reproducibly shown to associate with the "ARE-like" sequence in chromatin isolated from old rat hepatocytes (Figure 4.2D). Taken together, these results demonstrate that Nrf2-ARE3 complex formation is inhibited in the aged rat liver, but there is an age-specific recruitment of Nrf2 to an alternate ARE promoter.

Aging induces transcriptional remodeling of the Gclc/ARE3 complex

We investigated next whether loss of Nrf2-ARE3 binding alone was responsible for the age-related decline in ARE-mediated transcriptional regulation of *Gclc*. Nrf2 must bind with a bZip partner factor to elicit transcription through the ARE. Because there is an age-related decline in Nrf2 binding to the ARE3 complex,

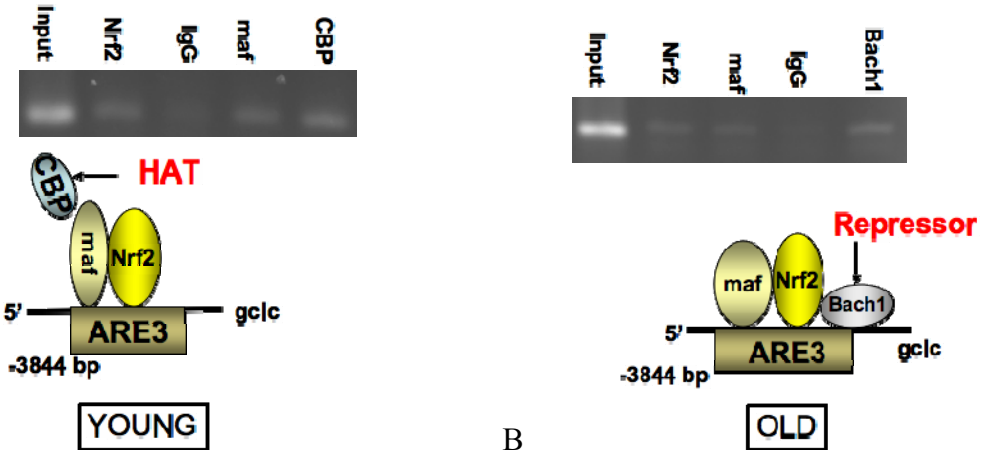


Figure 4.3 Aging induces a transcriptional remodeling of the Gclc ARE3 complex. (A) ChIP assays performed on hepatocytes from young rats show that Nrf2 binds to the *Gclc* ARE3 site with small maf proteins and the histone acetyltransferase, CBP. The presence of these transcription factors indicates an active *Gclc* transcriptome in the young. (B) In old rat hepatocytes, Nrf2 binds to ARE3 with small maf proteins and the repressor Bach1. The presence of Bach1 and the absence of CBP indicates a repressive environment at the ARE3, suggesting that this may be responsible for the age-related decline in *Gclc* transcription. Results are representative of ChIPs performed on hepatocytes from 3 young and 3 old rats.

we hypothesized that a Nrf2 partner protein that represses ARE-mediated transcription

may be responsible for the apparent promoter switching by Nrf2. In this regard, we utilized ChIP assays to determine the various partner proteins binding with Nrf2 to the ARE3 complex. Figure 4.3 A shows that in the young rat liver, along with Nrf2, small maf proteins, c-Jun and c-Fos bind and recognize the ARE3. Due to the current unavailability of a commercial antibody specific to either MafG, MafK or MafF, we were unable to identify the precise small Maf protein partnering with Nrf2. In addition, the histone acetyltransferase, CREB-binding protein (CBP), is also present on ARE3 (Figure 4.3A). While these ChIP assays cannot quantify the amount of a given transcription factor relative to each other because of differences in antibody-binding affinities, the data does indicate that the ARE3 in cells from young rats is transcriptionally active - only permissive binding partners with Nrf2 are present (see schematic under Figure 4.3A).

Using a similar strategy to map age-related changes to the ARE3, we found that the ARE3 transcriptome for *Gclc* in hepatocytes from old rats is significantly altered (Fig. 4.3B). First, chromatin from aged cells show the presence of Bach1. Several publications show that Bach1 is a repressor of ARE-mediated gene expression (164, 271, 272). Bach1 has a DNA-binding motif and associates with the ARE promoter when Nrf2 is limiting. Thus, it competes with Nrf2 for ARE binding. The presence of Bach1 at ARE3 suggests that *Gclc* expression may be repressed with age.

Secondly, there is a concomitant absence of the co-activator, CBP at the ARE3 promoter of old rats. CBP normally binds to the Neh2 domain of Nrf2 and maintains

an open chromatin configuration to facilitate ARE-mediated transcription (158, 167, 168). Again, the disappearance of CBP would indicate a decline in ARE-mediated expression. These changes, in aggregate, are interpreted to mean that a repressive transcriptional environment occurs with age at the *Gclc* ARE3 element. (See schematic under figure 4.3B). This is consistent with our data showing an age-related loss in Nrf2 binding to the ARE (Fig. 4.2B & C) and also reflects diminished *Gclc* gene expression shown previously. These data might also explain why Nrf2 might switch to a more permissive "ARE-like" promoter during aging, as an attempt to compensate for basal transactivation at the ARE3 locus.

Nrf2 Occupies the "ARE-Like" Promoter in Old Rats Along With AP1 Transcription Factors

We carried out further ChIP experiments to understand whether the age-specific formation of the Nrf2-ARE-like complex was accompanied by a permissive transcription factor profile. Figure 4.4A shows that in young rats, the "ARE-like" motif binds only c-Jun and c-Fos, but not Nrf2. This indicates that transcription through the "ARE-like" element may partially contribute to the expression of *Gclc* even in young animals. Jun-Fos acting as AP1 transcription factors, are known to weakly induce ARE-driven genes (142, 156, 157, 273).

Consistent with the observations made in young rat hepatocytes, c-Jun and c-Fos also associated with the "ARE-like" element in old rat hepatocytes;

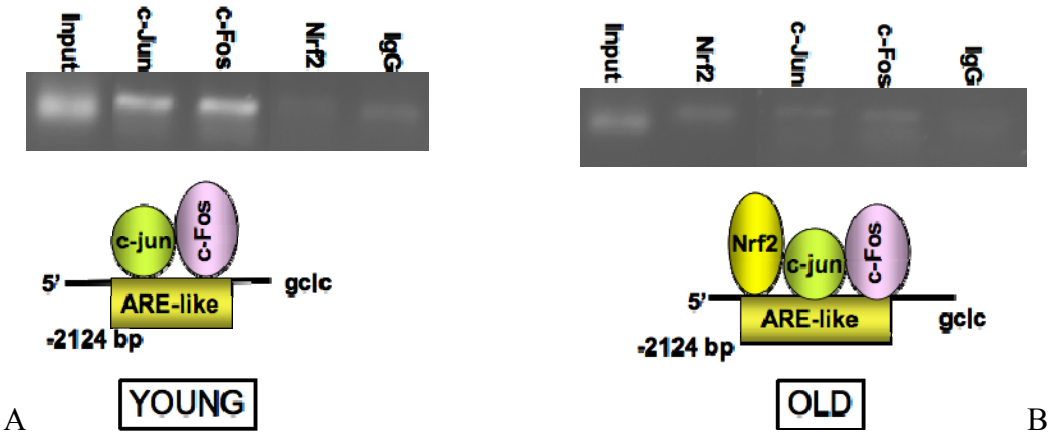
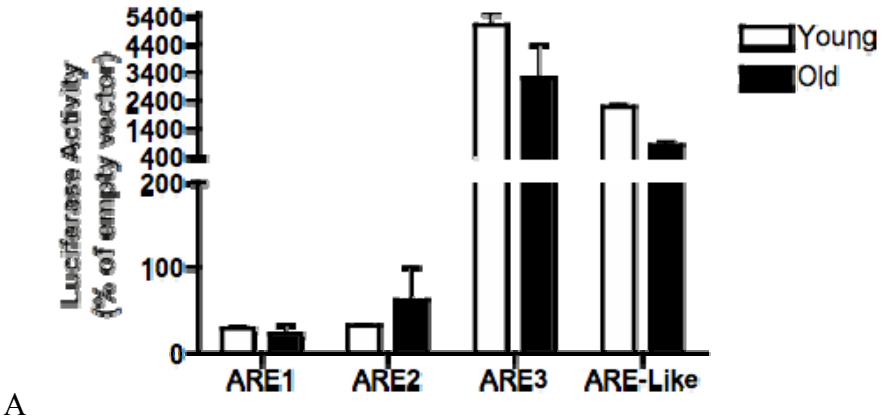


Figure 4.4 Transcription factors binding to the ARE-like element in young and old rats. (A) ChIP assays show that the AP1 transcription factors c-Jun and c-Fos bind to the ARE-like element in young rat hepatocytes. Immunoprecipitation with the Nrf2 antibody did not show any binding to the ARE-like sequence. (B) Nrf2 binds to the ARE-like element in old rat hepatocytes. ChIP assays indicate that c-jun and c-Fos bind to the ARE-like site with Nrf2. Results are representative of ChIP experiments from 3 young and 3 old rats.

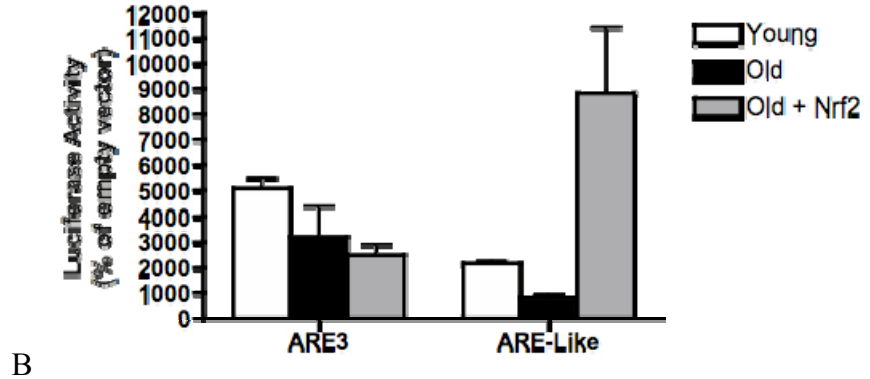
however, AP1 binding was also accompanied by Nrf2 associating with the "ARE-like" element (Figure 4.4B). No evidence of Bach1 binding to this element was observed. Thus, only co-activating transcription factors and no repressors bind at the ARE-like site with age. Taken together, these findings indicate that a promoter spill-over by Nrf2 occurs during aging. This could be an attempt by the cell to partially compensate for diminished transcriptional activation at the normal ARE3 locus.

Nrf2 Promoter Switching In Old Rats Does Not Compensate For The Age-Related Decline In ARE-Mediated Transcriptional Activity

To examine the consequences of the age-specific changes in Nrf2 binding to both ARE3 and the "ARE-like" element, we transiently transfected primary hepatocytes from young and old rats with minimal promoter-luciferase reporter constructs containing three copies of either ARE3 (pGL4.23*Gclc*[3X]ARE3.luc2) or "ARE-like" (pGL4.23*Gclc*[3X]ARE-like.luc2) elements. To verify that an absence of Nrf2 binding at the ARE1 and ARE2 sites denoted silencing of those promoters, we also carried out transfection experiments with the ARE1 and ARE2 luciferase reporters. Additionally, the pHRL-CMV Renilla luciferase construct was co-transfected as a control for transfection efficiency. Data from these experiments show that the ARE1 and ARE2 promoters display no luciferase activity (Figure 4.5A). As expected, there is a significant 38 $\pm$ 10% decline in the luciferase activity of the ARE3 promoter with age, indicating that lower Nrf2 binding at this motif correlates well with transcriptional activity (Figure 4.5A). However, the luciferase activity of the "ARE-



A



B

Figure 4.5 Nrf2 promoter switching in the old rat liver does not compensate for the age-related decline in ARE transcriptional activity. (A) Age-associated loss in luciferase reporter activity of the ARE3 and ARE-like promoters. Primary hepatocytes in culture from young and old rats were transfected with a luciferase reporter construct containing three copies of either one of the ARE elements or the ARE-like sequence and luciferase activities determined after 24 hours. A renilla luciferase construct was co-transfected to normalize for transfection. (B) Nrf2 over-expression in old rat hepatocytes restores transcriptional activity of the ARE-like promoter alone. Primary hepatocytes in culture from old rats were transfected with 0.5 μg of pcDNA2.1Nrf2 along with the ARE3 or the ARE-like promoter. Results show that overexpression of Nrf2 does not affect transcriptional activity of the ARE3 promoter, but increases luciferase levels of the ARE-like promoter above levels seen in young control. Luciferase reporter activities are calculated as Firefly/Renilla and are normalized to the activities of the empty vector in young and old rat hepatocytes respectively.

like" promoter also exhibited an age-related decline to the same extent as seen in the ARE3 promoter (Figure 4.5A). Given the age-specific occupation of the ARE-like promoter by Nrf2, these results are somewhat surprising. Hence, we hypothesized that

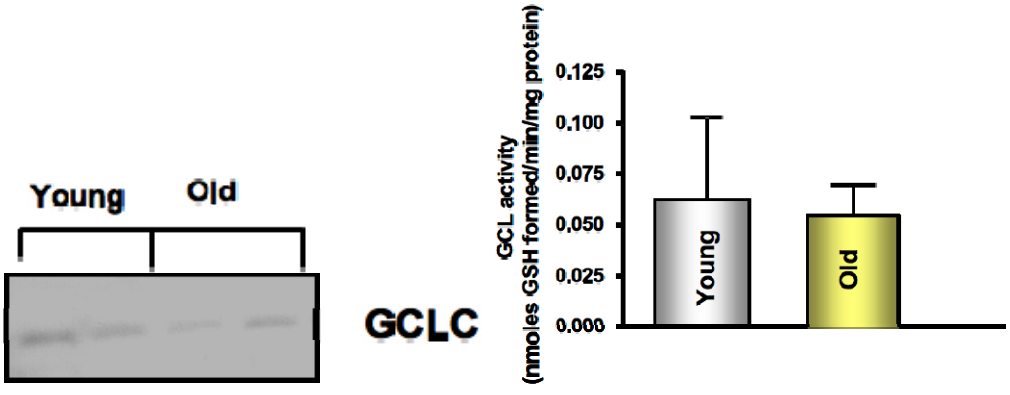
despite the presence of Nrf2 at the alternate ARE promoter, the levels of Nrf2 required to maintain normal Gclc expression through this promoter were limiting in the old rat liver.

To test this hypothesis, we co-transfected 0.5 μg of an Nrf2 expression plasmid along with the ARE3- and ARE-like- luciferase reporter constructs in hepatocytes from old rats. Figure 4.5B shows the results of Nrf2 over-expression on ARE3- and ARE-like luciferase activities in old animals compared with baseline activities in the young. An examination of the ARE3-driven luciferase activities shows that Nrf2 over-expression does not restore the age-related loss to the levels seen in the young. On the contrary, forced expression of Nrf2 fully reverses the age-associated decline in ARE-like activity. These results confirm that Nrf2 levels are limiting to transcriptional activity of the ARE-like promoter in the old rat liver. Additionally, these data also provide credibility to the hypothesis that loss in activity of the ARE3 site is governed by a two-pronged mechanism: lower Nrf2 binding as well as formation of a repressive transcriptional motif.

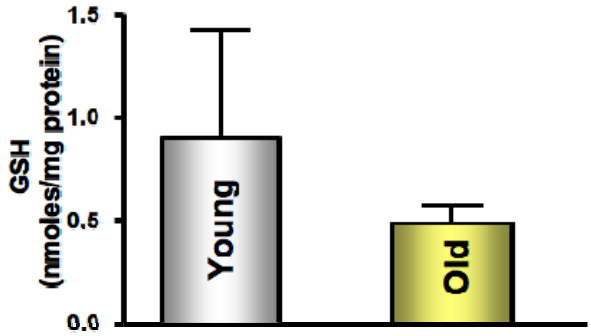
Lesion in Nrf2-ARE-mediated transcription contributes to age-related decline in GCL Activity and GSH Levels

We verified the consequences of an age-specific negative environment of the Nrf2-ARE regulon on hepatic GCLC protein levels, GCL activity and GSH levels. A western blot using total cell extracts from young and old rat hepatocytes shows that GCLC levels decline 42 $\pm$ 8 % ($P < 0.05$) with age (Figure 4.6 A). Furthermore, an examination of GCL activity showed a significant 33 $\pm$ 9 % ($P < 0.05$) loss in GSH

synthetic capacity (Figure 4.6 B). Lastly, we ascertained that old rats exhibited 40% lower total GSH levels, regardless of redox state (Figure 4.6 C). These results suggest that aberrant Nrf2-ARE-mediated transcriptional regulation in old rats contributes to the attenuated hepatic GSH content with age.



A **B**



C

Figure 4.6 Age-related decline in hepatic GSH synthesis. (A) GCLC protein levels decline with age in hepatocytes. Western analysis of GCLC shows a 42 + 8% loss in old rat hepatocytes compared to young. (B) Lower GCLC protein levels lead to decreased GCL activity (33%) in old rat hepatocytes. (C) Measurement of GSH levels in young and old rat hepatocytes show a significant 40% loss with age. Results are expressed as mean $\pm$ SEM.

4.5 Discussion

Aging result in hepatic GSH insufficiency as demonstrated by various laboratories (50, 94, 95, 104, 274). Although considerable effort has been devoted to understanding GSH homeostasis in aging, the transcriptional regulation of GSH synthesis has not been elucidated. We observed that the age-related loss in GSH synthetic capacity correlates with lower nuclear Nrf2 levels and binding to the ARE, a cis-acting sequence present in the 5'-flanking region of GSH-synthesizing genes. The

Nrf2-ARE regulon is a master transcriptional mediator of over 100 phase II and antioxidant defense genes and has been the subject of intensive investigations (83, 118, 119, 275, 276).

Our studies utilizing primary hepatocytes in culture from young and old rats demonstrate that nuclear Nrf2 itself is limiting in the elderly (Figure 4.1A) and this singularly represents the causal factor in down-regulation of ARE-mediated transcription during aging. Nuclear steady-state levels of Nrf2 determine the extent of transcription of target genes; hence, factors promoting nuclear localization are an intense topic of study currently (118, 119). However, this report only probed the mechanism of transcription at the Nrf2-ARE locus, so causes of Nrf2 nuclear translocation is beyond the scope of this paper. That being said, we demonstrate that the age-related decline of Nrf2 is the most striking in the nuclear compartment (Figure 4.1). Under basal conditions with no stimulation by inducers, relatively little Nrf2 translocates to the nucleus to maintain constitutive transcription of *Gclc* and other target genes. The fact that Nrf2 does not sufficiently build-up in nuclei of old rats despite an increased pro-oxidant environment in the aging cell indicates a fundamental dysregulation of Nrf2 activating pathways with age.

The other aspect governing transcription through the Nrf2-ARE nexus is the sequence of the ARE itself. Wasserman and Fahl postulated that transcription through the ARE core sequence (5'-GTGACnnnGC-3') is enhanced by certain nucleotides flanking the ARE, usually the presence of an embedded AP1 or a 5'-flanking AP1

element (125). Many phase II enzyme genes, including GCLC are characterized by the presence of multiple AREs in their 5'-flanking region (125, 131). Studies looking at the transcription of human GCLC, hemeoxygenase 1(HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GST) show that only one of the multiple ARE elements is transcriptionally active (277). The active ARE element in these genes has been shown to fulfill Wasserman and Fahl's criteria for ARE sequences exhibiting maximal basal and inducible transcription (125, 277). Of relevance to the present study, only one (ARE3) of the three AREs present in the rat *Gclc* 5-flanking region demonstrated Nrf2-binding activity (Figure 4.2). As expected, rat *Gclc* ARE3 fulfills the structural requirements of Wasserman and Fahl's active ARE sequence criteria (125). More specifically, the ARE3 has a 5'-flanking AP1 element that contains significant basal transcriptional activity (see chapter 2, Figure 2.3).

The major results of studies examining the mechanism of transcriptional regulation by Nrf2 reveal two important features: Nrf2 must form a heterodimer with a bZip protein to direct transcription through the ARE and, the ratio of Nrf2 to transcriptional activators and repressors determines ARE-mediated activation or silencing of target genes (275, 276). In the present study, a detailed analysis of Nrf2 and interacting transcription factors in hepatocytes from young rats showed that Nrf2 is bound to the active ARE3 element of *Gclc* in a complex with partners permitting transcription. However, aging shifts the active ARE3 complex to an inhibitory motif in terms of three significant characteristics: 1) lower Nrf2 binding, 2) non-recruitment of

the co-activator CBP to the ARE3 complex, and 3) presence of the chromatin modeling protein, Bach1.

It can be speculated that the latter two features are a consequence of lower steady-state levels and binding of Nrf2 to the ARE3. For example, the Neh2 domain of Nrf2 has been shown to recruit CBP to the ARE; hence, Nrf2 levels could be rate-limiting for CBP enrichment at ARE3 with age (158). CBP is a histone acetyltransferase and its absence at the ARE3 complex is expected to significantly inhibit chromatin opening for transcription (168, 278). Interestingly, CBP protein levels have been shown to decline with age in the liver, kidney, and cerebral cortex of rats (279, 280). Therefore, the age-specific absence of CBP at the ARE3 promoter observed in this study could be the direct result of lower CBP protein levels.

On the other hand, the presence of Bach1 can silence ARE-mediated transcription (166, 281, 282). Dhakshinamoorthy and Jaiswal showed that Bach1 occupies the ARE promoter in the NQO1 gene under conditions when Nrf2 nuclear translocation is not induced (164). Furthermore, Yoshida and co-workers showed that Bach1 forms heterodimers with small maf proteins when Nrf2 is limiting (283, 284). Secondly, they demonstrated that the Bach1-small maf complex acts as a potent architectural chromatin remodeling agent by forming DNA loops and inhibiting transcription through the ARE (283). These results suggest that the balance between Nrf2 and Bach1 on the ARE3 determines the extent of *Gclc* transcription. Thus the appearance of Bach1 at the ARE3 promoter during aging may alter chromatin

modeling in a negative manner and render the ARE3 inaccessible to other transcription factors, including Nrf2. Indeed, Bach1 has been shown to exert NFE2 promoter silencing during stages of erythrocyte development (285). Further experiments involving chromatin immunoprecipitation coupled with pharmacological or siRNA-mediated inhibition of Bach1 will be needed to assess the nature of this chromatin modification, which is outside the scope of the present paper. This could potentially explain why Nrf2 over-expression failed to significantly failed to up-regulate ARE3 transcription in old rat hepatocytes in this report (Figure 4.5B). Hence, the absence of CBP and the presence of Bach1 has the potential to significantly hamper ARE3-mediated transcription in old rats, as shown in Figure 4.5A.

A surprising finding of this study is that along with ARE3 binding, Nrf2 also binds to a response element resembling the ARE in old rats. This is an age-specific phenomenon, as no Nrf2 binding was detected to the "ARE-like" element in young rat hepatocytes. This apparent promoter spill-over of Nrf2 with age may be the result of a repressive complex formation with age; further investigations are underway to explore the precise role of Bach1 in this age-specific switch. As shown in Figure 4.2A, the ARE-like element contains the proximal core of the ARE, but the "GC" nucleotides required for induction are replaced by "AT". As a result, it has the potential to bind Nrf2, but not the small maf proteins that require the "GC" motif. While the consequences of activating this unique ARE-like sequence are unknown, further data examining relevant Nrf2 binding partners suggest that this ARE-like sequence may be active. Only potential AP1 co-activating partners and no repressors were found to bind

at this site. However, assessment of transcription in young and old rat hepatocytes using a luciferase reporter construct containing the ARE-like promoter revealed an age-dependent decline in activity (Figure 4.5A). Thus, an apparent attempt by the aging liver to compensate for diminished transcriptional activation of the normal ARE locus does not maintain GCLC and GSH levels (Figure 4.6).

To pinpoint whether lower age-specific enrichment of Nrf2 at the ARE3 locus is cause or consequence of the repressive ARE3 transcriptome, we induced a forced expression of Nrf2 in hepatocytes from old rats and checked for ARE3 and ARE-like transcriptional activity. Since high Nrf2 levels restored normal transcriptional activity to a greater extent at the ARE-like site and not the ARE3 site, these results suggest that the "ARE-like" site is comparatively more accessible to Nrf2 in the aging liver. Of relevance to other studies examining transcriptional regulation during aging, the partial promoter switching of Nrf2 has literature precedent from other systems. For example, Iakowa et al showed recently that aging induces growth arrest of proliferative pathways in the liver by a C/EBP alpha promoter-switching mechanism (286). Similarly, there are numerous reports of promoter silencing by methylation-dependent mechanisms during aging (287-289).

In summary, we have identified for the first time, a novel Nrf2-mediated promoter switching mechanism responsible for the age-related decline in GSH synthetic capacity. Other than an age-associated decline in GSH-synthesizing genes, results from our laboratory have demonstrated lower expression of other phase II and

antioxidant genes like GST2A, HO-1 and NQO1 (B.M. Dixon, personal communication). Whether a similar alternate ARE site exists in the above-mentioned gene promoters and the associated 'ARE-switching mechanism' occurs with age is the subject of ongoing investigations. Thus, the understanding of the molecular basis of GSH loss in the elderly is an important step in the development of therapeutic interventions to replenish GSH levels and maintain detoxification capacity of the liver with age.

Chapter 5

Lipoic Acid Remediates the Age-Associated Loss in γ-Glutamate Cysteine Ligase By Increasing Transcription Through A Novel Antioxidant Response Element

Swapna V. Shenvi and Tory M. Hagen

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5.1 Abstract

Aging leads to a decline in hepatic glutathione (GSH) levels, but treatment with the dithiol, *R*-(α)-lipoic acid (LA) reverses this loss by inducing the catalytic subunit of glutamate cysteine ligase (GCLC), the rate-controlling enzyme in GSH synthesis. We previously showed that, in old rats, LA increases the activity of Nrf2, a transcription factor that regulates *Gclc* expression. To study the precise mechanism how LA replenishes Nrf2-mediated GCLC levels in aging, primary hepatocytes in

culture from old rats were treated with various doses of LA. Results show that LA increases Nrf2 nuclear availability and transcription through the age-repressed antioxidant response element 3 (ARE3) promoter in the 5'-flanking region of *Gclc*; however, this LA-induced increase only partly compensates for the age-related decline in ARE3-mediated transcription. Rather, LA also induces Nrf2 enrichment and promoter activity to a significantly greater extent at an alternate "ARE-like" site that is unmasked during aging. With age, there is a loss of activating partners and the presence of a repressor, Bach1 binding with Nrf2 to the ARE3 site. Experiments to determine if LA treatment de-repressed Nrf2-mediated transcription by limiting Bach1 binding and/or enhancing permissive bZip factors to partner with Nrf2 show that LA did not act by reversing deterimental changes to the ARE transcriptome. Mutation analysis of the ARE3 and ARE-like element shows that the ARE-like element is more important for the LA-mediated induction of GCLC levels. Collectively, these results demonstrate that LA reverses the age-related loss in hepatic GCLC levels by increasing Nrf2-mediated transcription through a novel age-specific ARE promoter.

5.2 Introduction

One of the hallmarks of aging is a reduced capacity of cellular homeostatic mechanisms that protect the body against a variety of oxidative, toxicological, and pathological insults (9, 243, 290). This loss is most prominently exemplified in the age-related decline in hepatic glutathione (GSH) levels, as reviewed in (50) . GSH is the principal low molecular weight thiol antioxidant and the co-substrate for a variety of antioxidant and anti-xenobiotic (Phase II) enzymes (14, 52, 53). Decline in

constitutive GSH levels potentially leaves the cell susceptible to a number of internal and environmental stresses (227, 244). Conversely, increasing GSH steady-state levels confers enhanced protection against endogenous and exogenous insults (4, 10, 116, 291). In aging, however, when basal levels of oxidative stress become elevated, GSH synthesis does not concomitantly increase but actually declines, especially in the liver (94, 102, 109).

The synthesis of GSH from its constituent amino acids involves the actions of two ATP-dependent enzymes, γ-glutamylcysteine ligase (GCL) and GSH synthetase (191). GCL, the rate-controlling enzyme in the overall pathway, is a heterodimer composed of a catalytic (GCLC; 73 kDa) and a modulatory (GCLM; 30 kDa) subunit. GCLC retains all of the catalytic activity; GCLM improves catalytic efficiency by lowering the *K*m for glutamate and increasing the *K*i for GSH (192). Recently, we showed that the age-related loss of hepatic GSH is due to a decline in its synthetic capacity, in particular, activity and subunit levels of the GCL protein (94, 95). Furthermore, we demonstrated that GCL decline corresponded to both an age-associated insufficiency of nuclear levels of the transcription factor, NFE2-related factor 2 (Nrf2) and also its binding to the antioxidant response element (ARE) present in the 5'-flanking region of GCLC and GCLM genes (94).

The mechanism of dysregulation of ARE-mediated transcription of Gclc during aging is complex. Data from our laboratory has shown that along with lower Nrf2-ARE binding, aging induces a negative chromatin configuration at the normally

active ARE3 site present in the *Gclc* promoter (see chapter 4). This is accompanied by an age-specific shift of Nrf2 binding to a novel ARE-like upstream sequence in the *Gclc* promoter, possibly as a compensatory mechanism. However, these age-related changes in the ARE transcriptome fail to maintain GCLC and GSH levels during aging (chapter 4) (94).

Under unstressed conditions, a small amount of Nrf2 constantly translocates to the nucleus to maintain basal transcription of GCLC and a host of over 100 phase II and antioxidant defense genes (292-295). Nrf2 that is not needed for transcriptional activation is targeted for degradation in the cytosol by a cysteine-rich protein, Keap1 that bridges the transcription factor to a Cul3-based ubiquitin ligase (150, 152, 296-298). The Nrf2-ARE regulon, however, is highly inducible by electrophilic, oxidative and xenobiotic stimuli. A diverse class of chemicals including dithiols, alkylating agents, and electrophilic compounds can affect Nrf2 entry into the nucleus either by direct interaction with Keap1 or through activation of upstream kinases that phosphorylate Nrf2 (120, 275, 299-303). Thus, nuclear translocation greatly increases the half-life of Nrf2, making it available for transcriptional regulation of target ARE genes (154, 304).

We recently demonstrated that intraperitoneal injection (40 mg/kg body weight) of the dithiol, R-(α)-lipoic acid remediates the age-associated loss of hepatic GSH synthesis through an increase in nuclear Nrf2 levels and subsequent binding to a consensus ARE, showing that the inducible Nrf2-mediated transcriptional activity was

still intact in old rats (94). LA potentially stimulates nuclear Nrf2 availability through two theoretical mechanisms: modifying redox-active thiols on Keap1 because of the high electron density of its dithiolane ring; and /or by activating various upstream kinases (e.g. PKC), which has the ability to phosphorylate Nrf2 (305). However, the consequences of LA treatment on the ARE regulon have not been reported to date. For example, the balance of Nrf2 binding partners strongly influences whether transcription is increased (e.g. c-Jun, small maf) or diminished (e.g. Bach1, small maf heterodimers) (121). Whether LA alters this transcription factor composition still remains unanswered. In the present study, we asked whether LA reversed the age-related loss of hepatic GCLC levels strictly through a Nrf2-mediated mechanism. To answer this question, we looked at the consequences of LA treatment on the components of the active Gclc ARE3 complex. Furthermore, we also assessed whether LA worked through the ARE3 complex or the alternate "ARE-like" promoter in terms of Nrf2 binding and transcriptional activity during aging (see chapter 4).

Results show that LA increases nuclear steady-state levels of Nrf2 in a time- and dose-dependent manner in cultured hepatocytes taken from young and old rats. Additionally, LA promotes Nrf2 binding more significantly to the "ARE-like" site versus the typical ARE3 site. This may be because LA is unable to alter the negative transcription factor profile at the ARE3 site characterized by the absence of the co-activator CBP and the presence of the Bach1 repressor. Lastly, LA treatment reversed the age-associated loss of GCL activity and GSH levels. These results show that the

restoration of GSH synthetic enzymes during aging by LA is solely through a Nrf2-ARE mechanism and not through alterations in heterodimer partners of Nrf2.

5.3 Materials and Methods

Chemicals and antibodies

LA was a gift of Asta Medica (Frankfurt, Mainz, Germany). Restriction enzymes and T4 DNA ligase for subcloning were from New England Biolabs (Boston, MA). The dual-luciferase reporter assay system and reporter plasmids, pGL4 minimal promoter vector and phRL-CMV vector were from Promega (Madison ,WI).

Expression vector for Nrf2 (pcDNA3.1-Nrf2) was a kind gift provided by Dr. Jaiswal at Baylor College of Medicine, Houston, Texas (205). Custom oligonucleotides used in PCR cloning, subcloning, and DNA sequencing were purchased from Invitrogen (Carlsbad, CA). Sequence service was provided by CGRB at OSU. Rabbit anti-Nrf2 (H-300), anti-lamin B1, small maf antibodies and Nrf2 siRNA and scrambled sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other antibodies were purchased from the following suppliers: c-Jun and Bach1 (Abcam), c-Fos (eBioscience), CBP (Upstate Biotechnology, New York), and IgG (Calbiochem). Rabbit polyclonal antibody to GCLC was from Neomarkers. Protein A-Sepharose was purchased from Sigma. All high performance liquid chromatography solvents were HPLC grade reagents from Fisher Scientific. All other chemicals used were at least analytical grade.

Animals

Rats (Fischer 344, virgin male, outbred albino), both young (2–5 months: n =12) and old (24–28 months, n=25; National Institute of Aging animal colonies), were acclimatized in the Oregon State University animal facilities for at least 1 week before experimentation. Animals were maintained on a standard chow diet, and food and water were given ad libitum.

Hepatocyte Isolation and Culture

Rats were anesthetized using diethyl ether (Fisher Sci., Fair Lawn, NJ) and the liver was exposed by a midlateral incision in the abdomen. Rats were sacrificed by

cutting through the diaphragm and severing the vena cava. The animals were consistently sacrificed between 9:00 to 11:00 AM to minimize differences in food intake and diurnal variability. Liver tissue from young (3–5 months) and old (20–24 months) rats were dispersed into single cells by collagenase perfusion (306). Cell number was assessed by using a hemocytometer, and viability was determined by trypan blue [0.2% (wt/vol) in phosphate-buffered saline (PBS)] exclusion. Viability was normally greater than 90% in both age groups.

Freshly isolated hepatocytes were plated on collagen-coated culture dishes in William's Medium E supplemented with 5% FBS, 1 mM dexamethasone, 100 ng/ml insulin, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin for 4 h in 5% CO2 at 37°C to allow attachment to the dishes. The medium was then replaced with fresh supplemented William's Medium E, and the cells were cultured for an additional 48 h before chromatin immunoprecipitation, transfection with the appropriate ARE-luciferase constructs or LA treatments.

LA Treatments

Hepatocytes from young and old rats placed in primary culture were treated with various concentrations of LA for varied times to assess the stimulation of Nrf2 nuclear levels. Briefly, a 25–100 mM LA stock was prepared in 100% dimethylformamide (DMF). Cells were treated with vehicle (0.1% DMF) or 25, 50, 100 or 250 μM LA for up to 24 hours and nuclear extracts prepared. For chromatin immunoprecipitation or transient transfection of luciferase reporters, hepatocytes were

treated with 100 μM LA for 24 hours.

Preparation of nuclear extracts and Nrf2 analysis

Cells were scraped into ice-cold lysis buffer (10 mMHepes, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 10 mg/mL pepstatin A, 0.5 mM DTT, and 0.4% NP-40), incubated for 10 min, and centrifuged at 14,000 g for 3 min at 4 °C. The resulting nuclear pellet was resuspended in extraction buffer (20 mM HEPES (pH 8.0), 0.4 M NaCl, 1.0 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 10 mg/mL pepstatin A, and 10% glycerol), kept on ice for 15 min, and centrifuged at 14,000 g for 5 min at 4 °C. The supernatant containing the nuclear proteins was resolved by SDS-PAGE and Nrf2 levels determined by western blotting using Nrf2 antibody (1:1000) as described before.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation analysis was conducted using control rabbit IgG, anti-Nrf2, anti-small maf, anti-c-Jun, anti-c-Fos, anti-CBP, and anti-Bach1 antibodies. PCR primers are described in Table 3.1. 18 million hepatocytes were used for each chromatin immunoprecipitation. Briefly, proteins and DNA were cross-linked with 1% formaldehyde for 10 minutes at room temperature and cells lysed in SDS-lysis buffer containing protease inhibitors and then sheared to an average length of 500-800 bp by sonication using a Sonic Dismembrator Model F60 (Fisher Scientific, Pittsburgh, PA). Sheared chromatin was immunocleared with salmon sperm

DNA/protein A-sepharose and 10% of the precleared chromatin was stored and labeled as “input DNA”. The remaining chromatin was immunoprecipated with IgG (control) or Nrf2, small maf, c-Jun, c-Fos, CBP or Bach1 antibodies (2 μg) overnight at 4°C. Immune complexes were adsorbed onto salmon sperm DNA/protein A-sepharose beads. Immunoprecipitates were washed sequentially with wash buffers to reduce background. Protein–DNA complexes were eluted from the antibody with elution buffer (1% SDS, 0.1 M NaHCO3) and formaldehyde cross-links reversed by addition of NaCl (5 M) and heating at 65 °C overnight. DNA was purified using the QIAquick purification kit (Qiagen, Valencia, CA) and PCR performed using primer pairs that spanned each of the ARE and AP1 elements. PCR products were quantified using SYBR Green Mastermix (New England Biolabs) with the Mini Opticon 2 Real-Time PCR Detection System (Bio-Rad). Specific enrichment of DNA by anti-Nrf2 antibody was calculated by subtracting the PCR value of normal IgG from that of anti-Nrf2 antibody and by normalizing that value to the PCR input. Triplicate PCR reactions were conducted for each sample, and the experiments were repeated at least thrice. The specificity of the PCR products was confirmed by melting curve analysis and size (agarose gel electrophoresis).

Construction of luciferase reporter vectors

The ARE- and ARE-like-luciferase reporter plasmids were generated using the pGL4-minimal promoter vector (Promega) containing a minimal TATA promoter upstream of the firefly luciferase gene. The sequences of the inserts used in the

different plasmids are summarized in Table 2.1. Single-stranded oligonucleotides were first annealed to form double-stranded oligonucleotides and then ligated into the pGL4.23[minP] vector following the manufacturer's instruction (Promega). Each of the vectors was engineered by inserting 3 copies of each of the ARE elements present in the rat *Gclc* 5'-flanking region. The three different *Gclc* 25-bp ARE (3X)-driven luciferase reporter constructs [i.e., *pGL4.23Gclc*-3xARE1-Luc2, *pGL4.23Gclc*-3xARE2-Luc2, and *pGL4.Gclc*-3xARE3Luc2] and the *Gclc* ARE-like (3X)-driven luciferase reporter construct [Pgl4.23*Gclc*-3XARE-like-Luc2] were made by insertion of the appropriately hybridized complementary oligonucleotides [75 bp] with 2-bp overhangs into the *Xho*I/*HindIII* restriction sites of the pGL4-minimal promoter reporter vector. TOP10 competent cells were transformed with the recombinant DNA after ligation for amplification. After the plasmids were generated, the DNA sequence of the inserts was verified.

Hepatocyte transfection and luciferase assays

Reporter gene assays were used to determine the transcriptional activities of individual Gclc ARE elements and the ARE-like element in primary hepatocyte cultures from young and old rats and after LA treatments. Transient transfections were done in hepatocytes cultured on 6-well collagen-coated plates for at least 48 hours using the Effectene Transfection reagent (Qiagen, Valencia, CA). The cells were transfected with 1.6 μg of GCLC-Luciferase plasmids. Briefly, the DNA and 13 μl of Enhancer were dissolved in buffer EC from the kit to a total volume of 100 μl. The DNA–enhancer mixture was incubated at room temperature for 5 min. After

incubation, 5 μl of Effectene transfection reagent was added to the mixture, mixed, and incubated at room temperature for 10 min to allow transfection–complex formation. Medium (1 ml) was added to the mixture and mixed. The mixture was then immediately added to the well containing the cells and 1 ml of fresh medium. The total amount of plasmid DNA for transfection was adjusted by empty expression vector (pGL4.23). 0.02 μg of the control plasmid phRL-CMV encoding *Renilla* luciferase was included in each transfection to account for variability in transfection efficiency. In some cases, 0.5 μg of Nrf2 expression plasmid (pcDNA2.1 Nrf2) was co-transfected with luciferase reporter constructs. Thirty-six hours after transfection, cells were harvested with 1 x passive lysis buffer (Promega), and the supernatant was collected by brief centrifugation. Transcription activity was determined by the expression of firefly luciferase and was normalized to the renilla luciferase levels by using a dual luciferase reporter assay kit (Promega) on a Biolumat LB9505 luminometer (Berthold Detection Systems, Pfhorzeim, Germany). The means of at least three independent experiments, each carried out in duplicate, are shown with the $\pm$ S.E.

GSH Analysis and Measurement of GCL Activity

Briefly, cells were lysed in 10% perchloric acid (wt vol) containing 5 mM EDTA. After deproteinization, hepatic GSH and glutathione disulfide (GSSG) concentrations were determined according to the method of Fariss and Reed (245). To determine the amount of GSH bound to proteins, the acid-precipitated pellets were dissolved in 0.1 M phosphate buffer, and the pH was readjusted to 7.4 with 3 M KOH.

These samples were incubated in the presence of 10 mM DTT for 30 min at 37°C, and the amount of GSH released was determined by HPLC-UV analysis.

Hepatic GCL activity was detected as described (94). Briefly, hepatocytes were lysed in 0.25 M sucrose containing 1 mM EDTA, 20 mM Tris HCl (pH 7.4), and 1% (vol vol) protease inhibitor mixture P8340 (Sigma). The cytosolic protein fraction from crude homogenates was obtained by centrifugation and was subsequently filtered through microcon-10 (Millipore) tubes to remove endogenous inhibitors and substrates for GCL. GCL activity was initiated by adding protein (0.5 mg ml) to a reaction buffer containing 20 mM L-glutamic acid, 5 mM cysteine, 5 mM DTT, 10 mM ATP, 0.1 M Tris HCl (pH 8.2), 150 mM KCl, 20 mM MgCl2, 2 mM EDTA, and 0.04 mg ml acivicin. The samples were incubated in a water bath at 37°C for 45 min. Reactions were stopped by mixing 150 ul of the sample with an equal volume of 10% (vol /vol) perchloric acid. The amount of GCL formed was detected by using the same HPLC protocol for monitoring GSH levels. Quantitation was obtained by integration relative to a GCL external standard.

Statistical Analysis

The data are expressed as the means $\pm$ S.E. Statistical analysis was performed with the GraphPad Prism software version 3.03 (GraphPad Software Inc., San Diego, CA). We used a two-tailed Student's *t* test to compare the luciferase activity of individual *Gclc* promoter constructs. A *P* value less than 0.05 was considered to be significant. One-way analysis of variance (ANOVA) was used when multiple

comparisons were made, followed by Tukey's post-hoc analysis for multiple comparisons to a control.

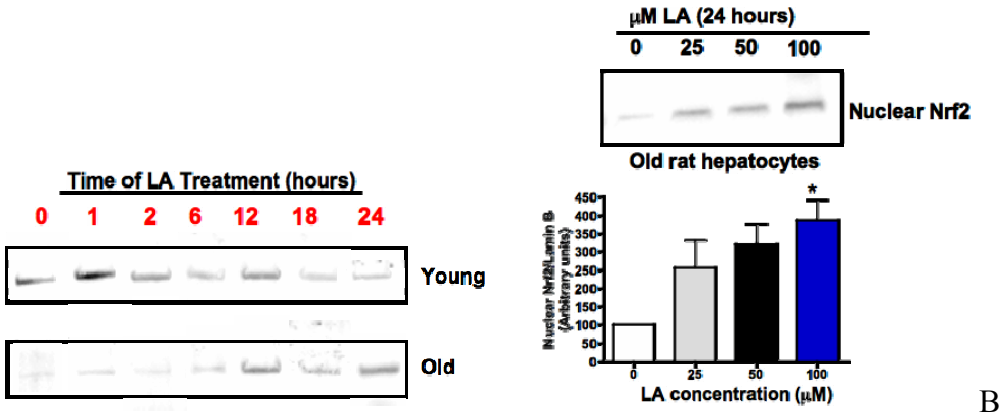
5.4 Results

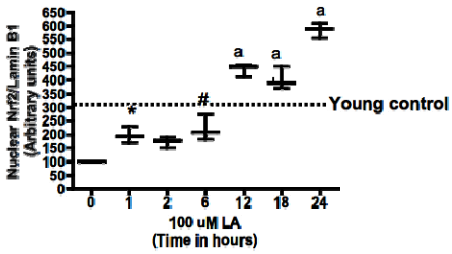
LA Induces Nuclear Levels of Nrf2 In A Time- and Dose- Dependent Manner in Hepatocytes From Old Rats

We previously showed that an acute intraperitoneal injection with 40 mg/kg LA to old rats increases hepatic nuclear Nrf2 levels within 12 hours, such that they are similar to nuclear Nrf2 content observed in young rats (94). In this study, we used

isolated hepatocytes in primary culture that maintain their aging phenotype to establish the range and timing of LA doses required to elicit the nuclear Nrf2 content required for normal ARE-mediated *Gclc* transcription. Accordingly, old rat hepatocytes were treated with LA concentrations ranging from 25-100 uM or DMF vehicle for up to 24 hours. Results show that LA treatment in vitro significantly increases nuclear Nrf2 within 1 hour of treatment in old rat hepatocytes, but Nrf2 content equal to that in young hepatocytes is not reached until 12 hours of treatment (Figure 5.1 A). To discern whether the LA-mediated activation of nuclear Nrf2 was dose-dependent, we checked nuclear Nrf2 levels in old rat hepatocytes 24 hours after treatment with 3 different concentrations of LA. Figure 5.2 B shows that there is a significant dose-dependent increase from 25-100 μM LA treatment in terms of Nrf2 nuclear localization. It should be noted that the 25-100 μM LA concentration range is similar to physiological hepatic concentrations achieved after LA administration.

LA Treatment In Old Rat Hepatocytes Stimulates Nrf2 Binding to GCLC ARE3, But Does Not Alter Nrf2 Heterodimer Partner Binding





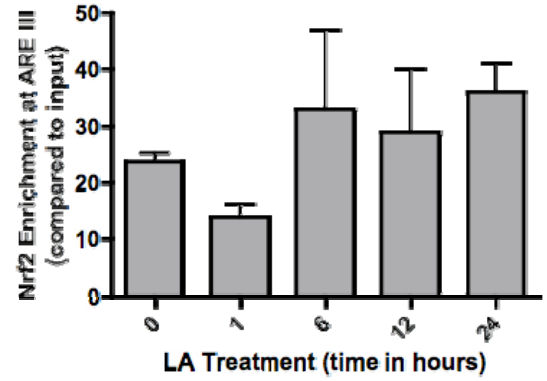
A

Figure 5.1 LA treatment induces nuclear steady-state levels of Nrf2 in a time-and dose-dependent manner. (A) Hepatocytes in primary culture from young and old rats were treated with 100 μM LA for up to 24 hours and nuclear Nrf2 content determined by Western blot. Results show a significant increase in nuclear Nrf2 levels in both young and old rat hepatocytes 1 hour after LA treatment. The dotted line in the graph indicates nuclear Nrf2 content in unstimulated young rat hepatocytes. *, $P < 0.05$; #, $P < 0.01$; and a, $P < 0.001$ compared to old control, respectively. (B) Old rat hepatocytes in primary culture were treated with 25-100 μM LA for 24 hours. Results show a dose-dependent increase in nuclear Nrf2 levels after LA treatment. *, $P < 0.05$ compared to old control. Results are representative of experiments performed on hepatocytes isolated from 3 young and 3 old rats and are indicated as mean $\pm$ SEM.

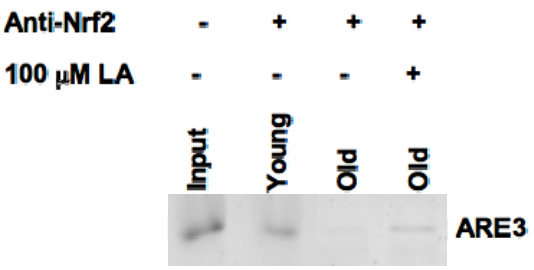
Data from our lab have conclusively shown that of the three ARE elements and an "ARE-like" element present in the 5'-flanking region of *Gclc*, only the ARE3 element exhibits Nrf2 binding (see chapter 2). However, during aging, a negative regulatory complex characterized by low Nrf2, absence of the co-activator CBP and presence of the repressor Bach1 is formed at the ARE3 site (see chapter 4). Therefore, we asked the question whether LA restores *Gclc* transcription through the ARE3 site by enriching Nrf2 or by affecting activation of CBP and/or removal of Bach1 from the

ARE3 complex. To answer the question whether LA enriched Nrf2 at the ARE3 site, we treated hepatocytes from old rats with 100 μM LA for 1, 6, 12 and 24 hours and monitored Nrf2-ARE3 binding using chromatin immunoprecipitation and qPCR to amplify the ARE3 fragment. Data shown in Figure 5.2 A demonstrate that LA increases Nrf2-ARE3 binding in a time-dependent manner and is maintained for up to 24 hours after treatment. It should be noted that Nrf2-ARE3 levels seen in the young are achieved by 6 hours of LA treatment in old rat hepatocytes. Figure 5.2 B is an agarose gel representation of replenishment of Nrf2-ARE3 binding after 24 hours of LA treatment in old rat hepatocytes. The basal level of Nrf2-ARE3 binding in young hepatocytes is included for a comparison.

Additionally, we investigated whether LA treatment caused an appearance of CBP at the ARE3 promoter or lowering of Bach1. Our results show that LA does not induce CBP or affect Bach1 binding to the ARE3 (data not shown), underlining the fact that LA-upregulation of *Gclc* during aging is solely mediated through Nrf2.



A

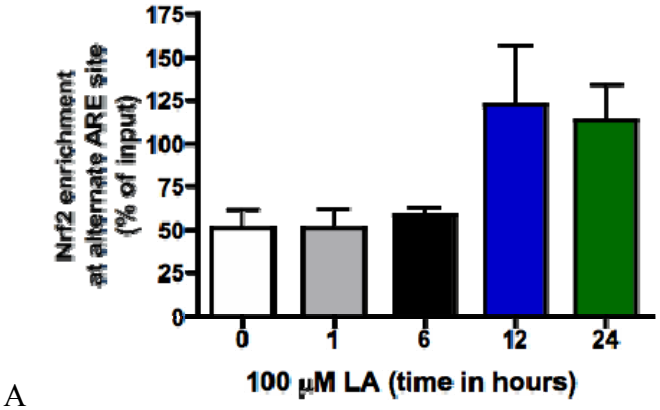


B

Figure 5.2 LA treatment in old rat hepatocytes stimulates Nrf2 binding to Gclc ARE3. (A) Primary hepatocytes in culture from old rats were treated with 100 μM LA or vehicle (0.1% DMF) for up to 24 hours and ChIP assays performed with anti-Nrf2 antibody. PCR amplification of Nrf2 immunoprecipitates with primers spanning the Gclc ARE3 element shows that Nrf2 is enriched at the ARE3 promoter 6 hours after LA treatment and is maintained for up to 24 hours. (B) Agarose gel representation of Nrf2-ARE3 ChIPs performed on old rat hepatocytes after 24 hours of LA treatment. PCR was performed for 25 cycles of amplification. Input denotes 10% of total sheared DNA before immunoprecipitation with Nrf2 antibody. Baseline Nrf2-ARE3 binding in young rat hepatocytes is shown for comparison. Results are representative of LA treatments on hepatocytes from 3 old rats and are depicted as mean $\pm$ SEM.

LA Treatment Stimulates Nrf2 Enrichment To A Greater Extent At the "ARE-Like" Promoter Compared to the ARE3 Site

In conjunction with the changes in transcription factor binding at the ARE3 site of *Gclc*, aging induces an apparent promoter switching of Nrf2 from the ARE3 site to an upstream "ARE-like" sequence (see chapter 4). We postulate that this "ARE-like" sequence is more amenable to transcriptional stimulation by LA, especially because of the repressive transcriptional profile existing at the normative ARE3 site with age. To probe this hypothesis, we treated hepatocytes from old rats with 100 μM LA for up to 24 hours and measured Nrf2 binding to the "ARE-like" element using chromatin immunoprecipitation coupled to quantitative PCR. Results show that LA increases Nrf2 binding to the "ARE-like" element over time (Figure 5.3A). Furthermore, LA-mediated induction of Nrf2 at the "ARE-like" site was more potent than that compared to the ARE3 site, at all time-points (Figure 5.2A and 5.3A). Figure 5.3B illustrates that the amount of Nrf2 induced at the "ARE-like" site after 24 hours of LA treatment was 65 $\pm$ 6% ($P < 0.05$) higher compared to the ARE3 site. These data lead further credence to the proposal that Nrf2-ARE3 binding cannot be increased over a certain threshold in the aging liver because of a negative remodeling of the open chromatin configuration. We also did more ChIPs to determine whether LA altered the enrichment of c-Jun and c-Fos proteins binding to the "ARE-like" site with Nrf2 in the old rat liver. Our results show that LA does not affect the levels of these key AP1 proteins at the ARE- like element (data not shown). Taken together, these data indicate that LA specifically increases Nrf2 levels at the ARE promoters of GCL in the aging rat liver.



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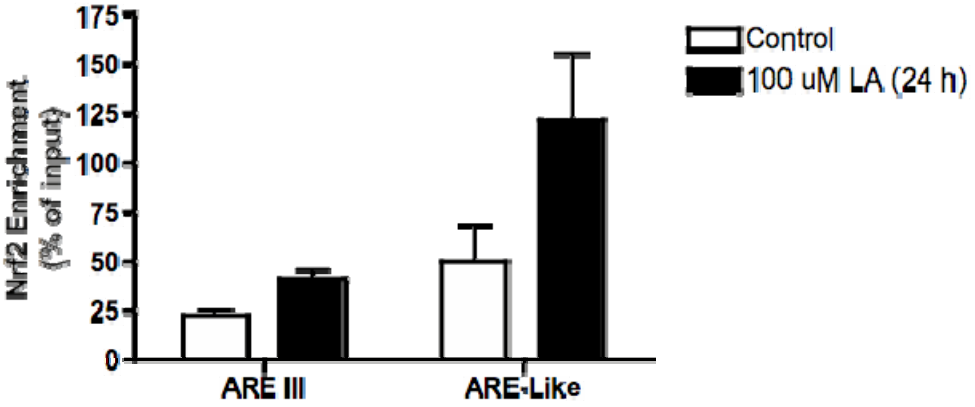


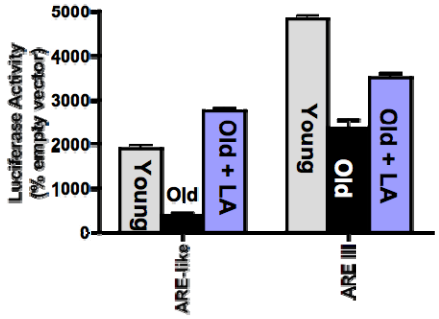
B

Figure 5.3 LA treatment stimulates Nrf2 binding to a greater extent at the ARE-like promoter compared to the ARE3 site. (A) Primary hepatocytes in culture from old rats were treated with or without 100 μM LA for up to 24 hours and ChIP assays performed to determine Nrf2 binding to an imperfect ARE site in the Gclc promoter. Results show that LA treatment increases Nrf2 enrichment at the ARE-like promoter after 12 hours and is maintained up to 24 hours. (B) Comparison of LA stimulation of Nrf2 binding to the ARE3 and the imperfect ARE promoter. Data from ChIP assays show that LA treatment for 24 hours significantly increases binding at the ARE-like promoter [65 $\pm$ 6% ($P < 0.05$)] compared to the ARE3 site. Results are depicted as mean $\pm$ SEM of ChIPs performed on hepatocytes from three old rats.

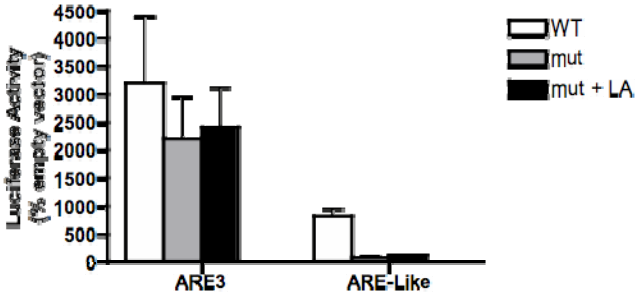
LA Treatment Completely Reverses The Age-Related Decline in Transcriptional Activity of The "ARE-like" Element, But Not the ARE3 Element

To determine whether transcriptional activities of the two ARE elements in the *Gclc* 5'-flanking region paralleled the induction of Nrf2-binding activity after LA treatment, we transiently transfected primary hepatocytes from old rats with luciferase reporter constructs containing three copies of the ARE3 or "ARE-like" elements and treated them with 100 μM LA for 24 hours. Measurement of luciferase reporter activities comparing unstimulated transfected cells to those after LA treatment show that the activities of both the ARE3 and "ARE-like" element increase under LA stimulation (Figure 5.4 A). Therefore, we questioned whether LA was an effective agent to restore the ARE activites lost during aging. A comparison of the LA-induced luciferase activities reveals that the transcriptional activity of the "ARE-like" element is restored to the levels seen in the young. However, despite an increase with LA stimulation, the luciferase activity of the ARE3 element does not reach the normal threshold seen in young animals (Figure 5.4 A), showing that LA is unable to overcome the partial chromatin silencing of the ARE3 regulon with age.

Based on the above data, we asked which of the two ARE elements was the precise target of LA-mediated restoration of hepatic *Gclc* expression in old rats. To answer this question, we mutated the purines to pyrimidines in the 'TGAC' core of the ARE3 and "ARE-like" elements. Results from the mutational analysis shows that the mutated ARE3 element retains 50% of its basal transcriptional activity, while the

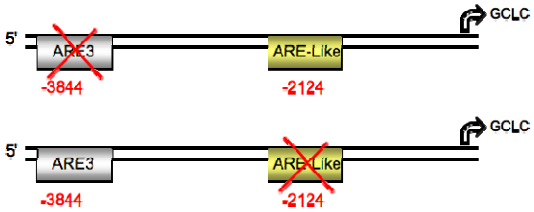


A



B

ARE3 : GTGAGGCGGCA
ARE3mut : GGTCTGCGGCA
ARE-like : CTGAATCATG A
ARE-like mut: CGTCCTCATGA



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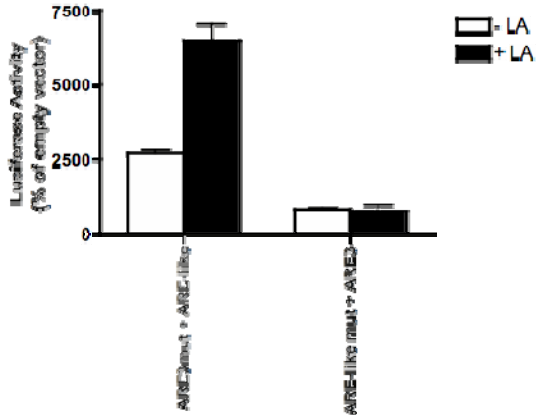

D

Figure 5.4 LA Treatment Completely Reverses The Age-Related Decline in Transcriptional Activity of The "ARE-like" Element, But Not the ARE3 Element. (A) Primary hepatocytes in culture from young and old rats were transfected with luciferase reporters containing three tandem copies of either ARE3 or "ARE-like" elements and were treated with 100 μM LA or vehicle (0.1% DMF) for 24 hours. Luciferase reporter assays show that LA increases transcriptional activity of both elements in old rat hepatocytes, but restores activity of only the "ARE-like" element to levels seen in the young. (B) Schematic representation of ARE3 and "ARE-like" mutants compared to wild-type. (C) Old rat hepatocytes in primary culture were transfected with ARE3 or "ARE-like" luciferase reporters or their mutants and treated with 100 μM LA or vehicle. Results from luciferase assays show that the transcriptional activity as well as response to LA is completely abolished by the mutation in the "ARE-like" element, but not the ARE3 element. (D) The ARE-like site is the target of LA stimulation. Primary hepatocytes in culture from old rats were co-transfected with an ARE3 mutant and a wild-type ARE-like element and vice-versa and treated with 100 μM LA or vehicle. Results show that LA-induced transcription is intact when the ARE3 mutant is transfected in the context of the ARE-like wild-type but not when the "ARE-like" element is mutated. Results are mean $\pm$ SEM of experiments performed on hepatocytes from three old rats.

mutated “ARE-like” element only displays 7% of its normal transcription (Figure 5.4 B). This discrepancy may be due to the fact that the ARE3 element has a 5’-AP1 flanking region that contributes to its basal activity. As expected, LA stimulation of the individual mutant ARE3 and “ARE-like” elements did not elicit an increase in transcription (Figure 5.4 B). Next, we co-transfected the mutated ARE3 element along with the normal “ARE-like” element and conversely, the mutated “ARE-like” element in the context of the normal ARE element, as shown in Figure 5.4 C. Results show that when the ARE3 element is mutated, luciferase activity is still upregulated by LA via the normal “ARE-like” promoter (Figure 5.4 D). However, when the “ARE-like” promoter is abolished, there is no significant upregulation of LA-mediated luciferase levels through the ARE3 (Figure 5.4 D). These data conclusively show that the novel age-specific “ARE-like” site is the target of LA stimulation during aging.

Age-Related Loss of GCL Activity and GSH Levels Is Reversed By Pharmacological Treatment With LA

To understand whether the LA-mediated increase in Nrf2 binding and activity of the ARE-like element translated into elevated GCL levels, we measured GCLC protein content in hepatocytes from old rats treated with 100 μM LA or vehicle. LA treatment reversed the age-related decline in GCLC levels within 12 h (1.8-fold increase; $P < 0.05$; Fig. 5.5 A) after treatment. As predicted by higher GCLC protein content, hepatic GCL activity and GSH levels also significantly increased after LA treatment. Heightened GCL activity was observed over the 24-h time course and directly correlated with the LA-induced increase in GCLC protein (Fig. 5.5B).

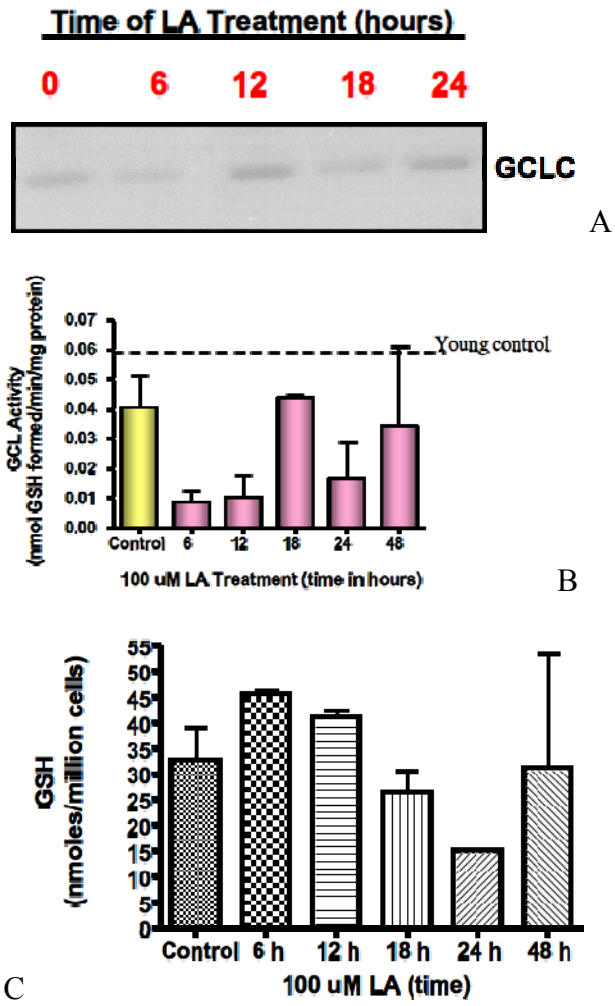

Figure 5.5 LA induces hepatic GSH synthesis in old rat hepatocytes. (A) Primary rat hepatocytes from old rats were treated with 100 μM LA for up to 24 hours and GCLC levels measured by western blot. Results show that LA induces GCLC levels 12 hours after treatment and is maintained for up to 24 hours. (B) Hepatic GCL activity is induced 18 hours after LA treatment. (C) LA treatment increases hepatic GSH levels 6 hours after LA treatment. Results are mean ± SEM of experiments performed on hepatocytes from 3 old rats.

Concomitant with heightened GCL levels and activity, hepatic GSH levels also increased and were actually higher than that seen in untreated young rat hepatocytes 24 h after LA treatment (Fig. 5.5C). These results demonstrate that LA reverses the age-related loss in GSH synthesis by activating transcription and Nrf2 binding to a novel ARE-like element in the 5'-promoter region of *Gclc*.

5.5 Discussion

In this paper, we present important evidence to show that LA reverses the age-related decline in hepatic GSH synthesis by influencing two intertwined mechanisms involved in *Gclc* transcription: i) by maintaining nuclear Nrf2 levels that are normally limiting during aging, and ii) by promoting Nrf2 binding and activity of a novel "ARE-like" sequence that is unmasked during aging in the *Gclc* promoter.

This study highlights LA as an effective means to maintain transcriptional mechanisms of GSH synthesis during aging. It has long been established that LA increases intracellular GSH levels in a variety of cell types and tissues by enhancing cysteine availability (95, 188). Recent evidence from our lab showed that LA affects intracellular GSH levels during aging by increasing transcription of GSH synthesizing enzymes (94). These results illustrate the power of LA to markedly augment endogenous cellular antioxidant capacity via increased ARE-mediated gene expression. This could arguably be a more important means to maintain cellular antioxidant defenses in times of oxidative insult than LA merely acting as a free radical scavenger, which has been proposed as the mechanism of action based on the antioxidant properties of LA *in vitro* (34, 183, 184, 266, 307). As GSH is the most abundant cellular antioxidant, but not directly available from the diet, the effect of LA is to increase antioxidant capacity to a degree that could never be achieved by an exogenous antioxidant alone.

The concentrations of LA used in the study were in the range of physiological concentrations achieved by consumption of dietary sources of LA (308). Endogenous

synthesis of LA solely supplies it for mitochondrial α-ketoacid dehydrogenases and this pool does not readily enter the cytosol (309). Results from both animal and human studies show that LA is readily bioavailable from the diet and can potentially activate Nrf2-mediated gene expression (310). However, it must be emphasized that nonprotein-bound LA neither accumulates nor remains at high levels in tissues. In fact, no detectable free plasma lipoate is evident in the post-fed state (308). These traits may actually make LA a better Nrf2-activating agent versus stronger compounds that elicit a sustained GCLC gene induction.

A potential reason why the atypical ARE sequence is targeted by LA may be due to the formation of an age-specific negative regulatory complex at the ARE3 site that is normally active in the young liver (Figure 4.3). Aging induces a loss of Nrf2-ARE3 binding leading to occupancy of the ARE3 site by Bach1 and an exit of the co-regulator CBP from the active transcriptional complex. Bach1 is a chromatin architectural protein (283, 284). Under conditions of low Nrf2 availability, Bach1 competes with Nrf2 for ARE binding and forms heterodimers with the small maf proteins (164, 311). This leads to the formation of closed DNA loops refractory to transcription. Similarly, low amounts of Nrf2 are insufficient to recruit CBP that binds to the Neh2 domain of Nrf2 and potentiates transcription through the ARE several-fold (158, 168). Hence, exit of CBP and presence of Bach1 delivers a ‘one-two punch’ to transcriptional regulation through the ARE3 element during aging. Consequently, despite the normal nuclear levels of Nrf2 achieved by LA stimulation, the closed chromatin configuration does not allow LA-mediated transcription through the ARE3

site.

On the other hand, there is no repressor complex present at the "ARE-like" site, facilitating Nrf2 binding. This may explain why LA is successful in inducing Nrf2 levels and activity of the "ARE-like" site during aging. Results from mutational analysis of ARE3 and the ARE-like element in Figure 5.4B & C also lend credence to the notion that the alternate ARE-element is the main site of LA-induced compensation of transcription during aging.

An important question that must be posed here is whether the consequences of activating this unique ARE-like sequence in aging are beneficial. Data presented in Figure 5.5 show that LA treatment maintains the GCL activity and GSH levels that decline in the aged liver, proving that transcriptional activation of the ARE-like element is advantageous in old rats and may be an epigenetic compensation during aging.

In summary, the evidence presented in this paper shows that in vitro treatment of lipoic acid to old rat hepatocytes in cell culture can mitigate the age-related loss of GSH synthesis through a previously unidentified novel transcriptional mechanism. In addition to contributing to our understanding of the aging process, these findings suggest new strategies to improve stress resistance in the elderly. Related phase II enzyme-inducing agents have not yet been advocated as a possible means to improve the morbidity and mortality associated with aging. In this study, we used GCLC as a

quintessential Nrf2-responsive, ARE-containing gene. As there are sequence variations for ARE in different genes, there is the possibility for differing degrees of *R*-LA remediation of Phase II detoxification response. Thus, it is necessary to discern the global age-related loss of Phase II detoxification gene expression and the degree that *R*-LA acts as an effective therapy to prevent this loss. The results presented herein strongly suggest that further research, in this regard, is warranted.

Chapter 6

Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid

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6.1 Abstract

Glutathione (GSH) significantly declines in the aging rat liver. Because GSH levels are partly a reflection of its synthetic capacity, we measured the levels and activity of γ-glutamylcysteine ligase (GCL), the rate-controlling enzyme in GSH synthesis. With age, both the catalytic (GCLC) and modulatory (GCLM) subunits of GCL decreased by 47% and 52%, respectively ($P < 0.005$). Concomitant with lower subunit levels, GCL activity also declined by 53% ($P < 0.05$). Because nuclear factor erythroid2-related factor 2 (Nrf2) governs basal and inducible GCLC and GCLM expression by means of the antioxidant response element (ARE), we hypothesized that aging results in dysregulation of Nrf2-mediated GCL expression. We observed an ~50% age-related loss in total ($P < 0.001$) and nuclear ($P < 0.0001$) Nrf2 levels, which suggests attenuation in Nrf2-dependent gene transcription. By using gel-shift and super-shift assays, a marked reduction in Nrf2-ARE binding in old vs. young rats was noted. To determine whether the constitutive loss of Nrf2 transcriptional activity also affects the inducible nature of Nrf2 nuclear translocation, old rats were treated with (*R*)-α-lipoic acid (LA; 40 mg/kg i.p. up to 48 h), a disulfide compound shown to induce Nrf2 activation *in vitro* and improve GSH levels *in vivo*. LA administration increased nuclear Nrf2 levels in old rats after 12 h. LA also induced Nrf2 binding to the ARE, and, consequently, higher GCLC levels and GCL activity were observed 24 h after LA injection. Thus, the age-related loss in GSH synthesis may be caused by dysregulation of ARE-mediated gene expression, but chemoprotective agents, like LA, can attenuate this loss.

6.2 Introduction

One of the hallmarks of aging is a reduced capacity of cellular homeostatic mechanisms that protect the body against a variety of oxidative, toxicological, and pathological insults (9, 86, 267). Nowhere is this loss more pronounced than in the age-related decline in hepatic glutathione (GSH) levels. GSH is the principal low molecular weight thiol antioxidant and the cosubstrate for a variety of antioxidant and anti-xenobiotic (Phase II) enzymes (110, 111, 244, 312). Decline in constitutive GSH levels adversely affects cellular thiol redox balance and potentially leaves the cell susceptible to a number of internal and environmental stresses. Conversely, increasing GSH steady-state levels and/or its rate of synthesis confers enhanced protection against oxidative insult (114, 129). Due to the central role of GSH in cellular protective mechanisms, the induction of enzymes required for its synthesis represents a key adaptive response to oxidative injury. In aging, however, when basal levels of oxidative stress become elevated, GSH and the enzymes from which it is synthesized do not concomitantly increase but actually decline in many tissues (112, 244, 312, 313). This lack of a cellular compensatory response to loss in GSH and the existence of a prooxidant state in aging cells suggest that the coordination of cellular antioxidant defenses may be altered with age.

The synthesis of GSH from its constituent amino acids involves the actions of two ATP-dependent enzymes, γ-glutamylcysteine ligase (GCL) and GSH synthetase. GCL, the rate-controlling enzyme in the overall pathway, is a heterodimer composed of a catalytic (GCLC; 73 kDa) and a modulatory (GCLM; 30 kDa) subunit. GCLC

retains all of the catalytic activity; GCLM improves catalytic efficiency by lowering the *K*m for glutamate and increasing the *K*i for GSH (191).

The basal and inducible expression of these GCL substituents seem to be mediated by means of the antioxidant response element (ARE) (79, 130, 147). The ARE is a cis-acting enhancer sequence that transcriptionally regulates Phase II detoxification enzymes, which are critical for maintaining cellular redox status and protecting against oxidative damage (118). A potential loss in GCL transcriptional regulation associated with aging may be indicative of a global decline in Phase II defense systems.

Recent studies show that nuclear factor erythroid2-related factor 2 (Nrf2) is the principal transcription factor that regulates ARE-mediated gene transcription (145, 146). Direct evidence for this finding is provided by the observed reduced basal expression of GCL and other Phase II detoxification enzymes in Nrf2-null mice (146, 314-316). These mice also display a marked increased susceptibility to toxicological insult (146, 314-316). We previously showed that aged animals also exhibit similar losses in steady-state GSH levels and vulnerability to toxins as shown for Nrf2-null mice (202, 317). These striking similarities between aging and Nrf2-null mice raise the question as to whether disruptions in Nrf2-mediated gene expression also occur during aging.

Thus, the aims of this study were to determine whether the age-associated decline in GSH status that we observed is due, at least in part, to the loss of Nrf2-dependent regulation of GCL expression. Moreover, previous studies show that thiol-reactive substances such as 3H-1,2-dithiole-3-thione (D3T), pyrrolidine dithiocarbamate (PDTC) (79), sulforaphane, and (*R*)-α-lipoic acid (LA) (244, 318, 319) act as potent chemopreventive agents that increase cellular GSH and Phase II response. Thus, we also sought to understand whether potential lesions in basal regulation of Nrf2 prevent its activation by the disulfide chemoprotectant, lipoic acid.

6.3 Materials and Methods

Chemicals

LA was a gift of Asta Medica (Frankfurt, Mainz, Germany). Rabbit polyclonal antibodies to GCL catalytic and modulatory subunits were used (111). Histone H1 antibody from Calbiochem (EMD Biosciences, San Diego) was used as a means to verify equal loading in all lanes for immunoblot analysis. Nrf2 antibodies were obtained from Santa Cruz Biotechnology. All high performance liquid chromatography solvents were HPLC grade reagents from Fisher Scientific. All other chemicals were reagent grade or the highest quality available from Sigma.

Animals

Rats (Fischer 344, virgin male, outbred albino), both young (2–5 months: n = 25) and old (24–28 months, n 25; National Institute of Aging animal colonies), were acclimatized in the Oregon State University animal facilities for at least 1 week before experimentation. Animals were maintained on a standard chow diet, and food and water were given ad libitum.

LA (40 mg/ml) was dissolved in 2 M NaOH containing 154 mM NaCl, and the pH was adjusted to 7.4 with concentrated HCl. LA solutions were sterile-filtered and made fresh each day of use. LA (40 mg/kg of body weight) was administered by i.p. injection. To reduce diurnal variations, animals were killed between 10:00 and 11:00 a.m. each morning.

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

GSH Analysis and Measurement of GCL Activity

Briefly, tissues were homogenized in 10% perchloric acid (wt/vol) containing 5 mM EDTA. After deproteinization, hepatic GSH and glutathione disulfide (GSSG) concentrations were determined according to the method of Faris and Reed (245). To determine the amount of GSH bound to proteins, the acid-precipitated pellets were dissolved in 0.1 M phosphate buffer, and the pH was readjusted to 7.4 with 3 M KOH. These samples were incubated in the presence of 10 mM DTT for 30 min at 37°C, and the amount of GSH released was determined by HPLC-UV analysis.

Hepatic GCL activity was detected as described (4). Briefly, tissues were homogenized in 0.25 M sucrose containing 1 mM EDTA, 20 mM Tris-HCl (pH 7.4), and 1% (vol/vol) protease inhibitor mixture P8340 (Sigma). The cytosolic protein fraction from crude homogenates was obtained by centrifugation and was subsequently filtered through microcon-10 (Millipore) tubes to remove endogenous inhibitors and substrates for GCL. GCL activity was initiated by adding protein (0.5 mg/ml) to a reaction buffer containing 20 mM L-glutamic acid, 5 mM cysteine, 5 mM DTT, 10 mM ATP, 0.1 M Tris-HCl (pH 8.2), 150 mM KCl, 20 mM MgCl2, 2 mM EDTA, and 0.04 mg/ml acivicin. The samples were incubated in a water bath at 37°C

for 45 min. Reactions were stopped by mixing 150 μl of the sample with an equal volume of 10% (vol/vol) perchloric acid. The amount of GCL formed was detected by using the same HPLC protocol for monitoring GSH levels. Quantitation was obtained by integration relative to a GCL external standard.

Western Blotting Analysis

Tissues were homogenized and processed as described for the analysis of GCL activity. An aliquot of tissue homogenate (30 μg of cytosolic soluble proteins) was used for determining GCL protein content by Western blotting as described (4). GCLC and GCLM were identified according to molecular weight markers. Relative densities of the bands were digitally quantified by using NIH IMAGE analysis software.

Real-Time PCR of GCLC and GCLM mRNA

A portion of each liver was excised and stored in RNALater (Ambion, Austin, TX) at 20°C and homogenized by using a Dounce homogenizer. Total RNA was isolated from both young and old rat livers (n= 2 and n= 4, respectively) by using an RNeasy Midi Kit (Qiagen, Valencia, CA). cDNA was prepared from 12.5 μg of total RNA per group by using SuperScript II (Life Technologies, Gaithersburg, MD) and oligo(dT) primers (Qiagen) in a 50 μl reaction. Semiquantitative real-time PCR with mRNA-specific primers spanning exon-exon boundaries was performed by using the DNA Engine Opticon II system (MJ Research, Waltham, MA). Specifically, 62.5 ng of each cDNA pool, 0.3 μM of each primer (GCLC-F, 5'-

GTCTTCAGGTGACATTCCAAGC-3'; GCLC-R, 5'-TGTTCTTCAGGGGCTCCAGTC-3'; GCLM-F, 5'-CTGCTAAACTGTTCATTGTAGG-3'; GCLM-R, 5'-CTATTGGGTTTTACCTGTG-3'; Qiagen) (where ''F'' indicates forward primers corresponding to the sense strand whereas antisense reverse primers are designated with an ''R'') and Finnzymes' DyNAmo Master Mix containing SYBR Green (MJ Research) was used for the PCR reaction according to the manufacturer's instructions. Samples were run concurrently with standard curves derived from PCR products, and serial dilutions were performed to obtain appropriate template concentrations. Lamin A/C (F, 5'-GGTGGATGCTGAGAACAG-3'; R, 5'-CTCCAGCTCCTTCTTATACTGCTCC-3'; Qiagen) was used as a control for RNA recovery and reverse transcription efficiency. GCLC and GCLM values were normalized to lamin A/C mRNA levels and expressed as arbitrary units. Agarose gel electrophoresis and thermal denaturation (melt curve analysis) were used to confirm specific replicon formation.

Total and Nuclear Nrf2 Levels

Liver tissue was homogenized (1:10) in RIPA Buffer [150 mM PBS containing 1% (vol/vol) Igepal CA630, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, and 5 μg/μl protease inhibitor mixture], pH 7.4, and 50 μg of protein was used for Western analysis of total hepatic Nrf2 levels (as described below). In other studies, nuclear extracts were prepared from liver tissue by the method of Dignam *et al* (246). Protein (40 μg) was loaded in each well of a precast 12% Tris-HCl polyacrylamide gel

(Bio-Rad). Separated polypeptides were transferred to nitrocellulose membranes (Amersham Pharmacia) and probed with anti-Nrf2 antibodies at a 1:2,000 titer. Chemiluminescent detection was done by an ECL Western Blotting Detection kit from Amersham Pharmacia.

Electrophoretic Mobility-Shift Assay (EMSA)

Transcription factor binding to the ARE was determined by using an EMSA. Nuclear extracts were prepared as described earlier. All gel-shift assays were performed for three sample replicates in each group. A synthetic double-strand oligonucleotide probe for the ARE (5'-TGG GGA ACC TGT GCT *GA*G TCA CTG GAG-3') (Santa Cruz Biotechnology) was end-labeled by using [ATP-^{32}P] (Amersham Pharmacia) and T4 polynucleotide kinase (Promega). Binding reactions containing equal amounts of protein (9 μg) and labeled oligonucleotide probes were performed for 20 min at room temperature in binding buffer (4% glycerol, 1mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris, pH 8.0). Specific binding was confirmed by using 100-fold excess unlabeled ARE oligonucleotide as a specific competitor. Protein–DNA complexes were separated by gel electrophoresis by using 6% nondenaturing polyacrylamide gels followed by autoradiography for 18 h to detect the degree of retardation produced by binding to the probe.

Supershift Assay

Binding of Nrf2 to the ARE was determined by supershift assays where anti-Nrf2 antibodies were incubated with the nuclear extracts at 4°C overnight before carrying out the EMSA reaction.

Statistical Analysis

The statistical significance between means of two independent groups was determined by Student's *t* test, assuming equal variances. For the comparison of treatment effects of LA in old rats, a one-way analysis of variance with Bonferroni's post hoc test was used. All of the results were considered significant if the *P* value was < 0.05. Statistical analysis was performed by using PRISM 4.0 software (GraphPad, San Diego).

6.4 Results

Age-Associated Changes in GSH Levels and GSH Biosynthetic Capacity.

Hepatic GSH levels were measured to establish the extent of age-related changes in this key antioxidant. Compared with young rats, old animals exhibited a significant 35% decline ($P < 0.05$) in overall GSH concentrations, regardless of redox state (Figure 6.1A). To ascertain whether this apparent loss of total GSH was due to formation of protein-GSH mixed disulfides, glutathiolation of proteins was measured. With age, the concentration of GSH bound to proteins increased by 40% from 11.6 $\pm$ 1.5 to 19.8 $\pm$ 5.2 pmol/mg of tissue ($P < 0.05$). Despite this increase, protein-bound GSH levels could not account for the age-dependent loss of total GSH.

To discern whether the age-related decline in hepatic GSH was due, in part, to diminished synthetic capacity, protein levels and activities of GCL were examined. Results show a 53 6% loss in hepatic GCL activity in old compared with young rats ($P < 0.05$; Fig. 6.1B). Because GCL is composed of both a catalytic and modulatory subunit, loss in activity could be due to a decline in either GCLM and/or GCLC content. To assess how aging affects the steady-state levels of these subunits, Western analysis was performed. Results show that GCLC levels in old rats were on average 47 $\pm$ 8% lower than that observed in young rats ($P < 0.005$; Fig. 6.1C); GCLM levels also exhibited a similar loss with age (52 $\pm$ 14%; $P < 0.005$; Fig. 6.1C). These results indicate that the attenuated hepatic GSH content in old rats is largely accounted for by lower levels and activities of GCL.

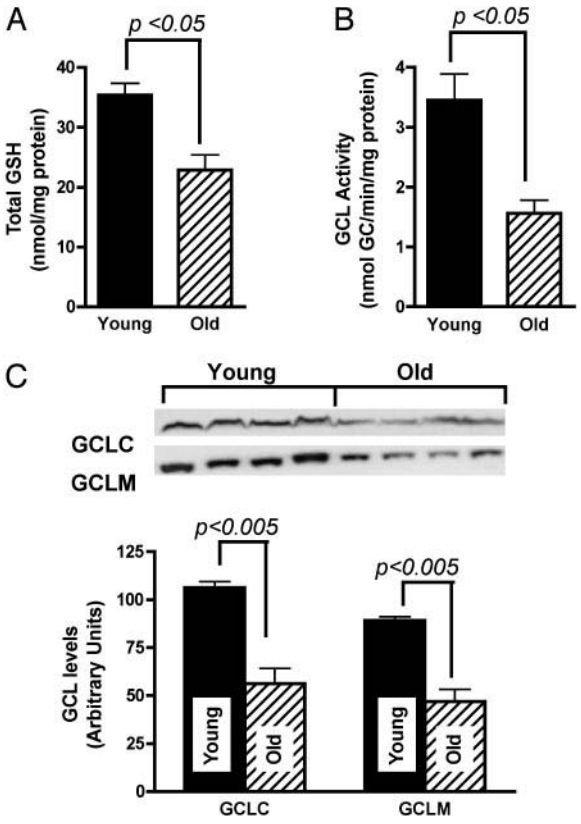

Figure 6.1 Age-related decline in total hepatic GSH is due to loss in GCL activity and expression. Hepatic GSH levels in young (3 mo; *n*=4) and old (24 mo; *n*= 4) F344 rats are shown in A. Results show a 35% decline in total GSH [GSH + 2 glutathione disulfide (GSSG)] in old relative to young rats. (B) Measurement of GCL activity reveals a significant 54.8% decline with age. Western analysis of GCL subunits shows that decreased GCL activity was, in part, due to the lower levels of GCLC and GCLM in old relative to young rats (*C*). Results are expressed as the mean $\pm$ SEM.

GCL Subunit mRNA Levels

Loss of GCL subunits could be due to an age-associated attenuation in GCLC and GCLM message levels as previously observed (4). To discern whether the loss in GCL subunits in this study were due to lower expression, semiquantitative real-time PCR was performed to monitor age-related changes in GCLC and GCLM message abundance. When normalized to lamin A/C, hepatic GCLC levels in old rats were 0.33 $\pm$ 0.06 a.u., which was ~41.1% lower than in young rats (0.56 $\pm$ 0.1 a.u). Similarly, steady-state levels of hepatic GCLM mRNA in old rats (1.1 $\pm$ 0.1 a.u.) were 30.0% lower than average values seen in young rats (1.5 $\pm$ 0.2 a.u.). These data suggest that the observed lower GCL activity and protein content may be due to diminished gene expression.

Age-Related Decline in Nuclear Nrf2 and Antioxidant Response Element Binding

Due to the central role of Nrf2 in regulating GCLC and GCLM gene transcription, we next examined whether cellular and nuclear Nrf2 levels are adversely affected during aging. Western blot assays showed that hepatic Nrf2 levels declined by 56 $\pm$ 2% (n=3 young; n=4 old; $P<$ 0.001) in old relative to young rats. Cell fractionation and analysis of nuclear extracts also revealed that basal nuclear Nrf2 levels decreased concomitantly (51 $\pm$ 7%) with this overall loss in hepatic Nrf2 ($P<$ 0.0001; Figure 6.2A). These results suggest that normal steady-state Nrf2 levels decline in the aging rat liver and affect basal Nrf2-mediated gene transcription.

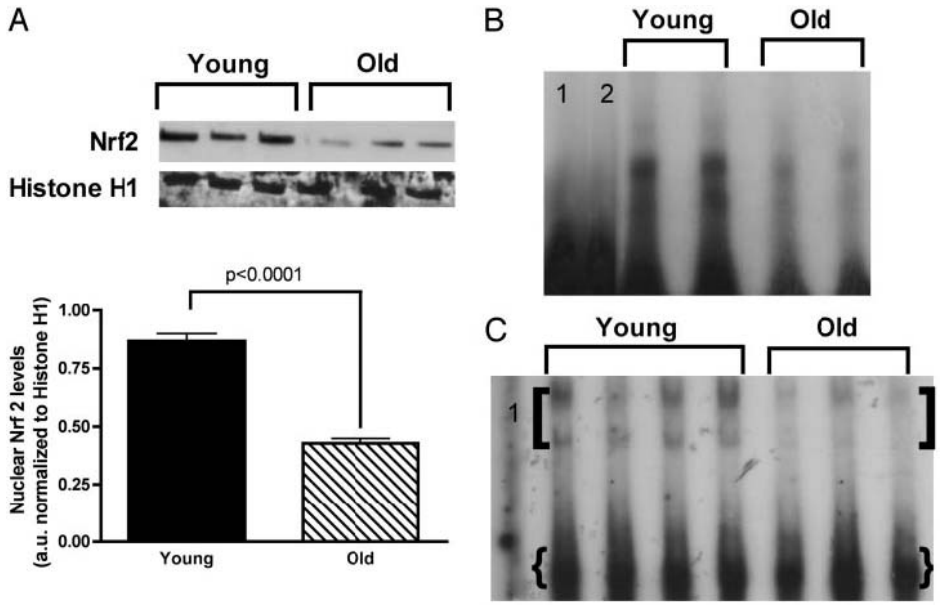

Figure 2 Aged rats display a significant loss in nuclear Nrf2 content and ARE-binding activity. Western analysis of Nrf2 present in nuclear extracts prepared from young and old rats are shown in A. Results are also graphically presented relative to Histone H1 loading controls and show that basal nuclear Nrf2 levels are 51% lower on an age basis. (B) EMSA analysis using nuclear extracts from young and old rats shows an overall age-related loss of transcription factor binding to the ARE consensus sequence. Lane 1 is the migration of free probe in absence of nuclear extract. A negative control using excess unlabeled cold probe is shown in lane 2. (C) Supershift analysis reveals that Nrf2 binding to the consensus ARE sequence declines with age. The two distinct supershifted bands are denoted by brackets whereas the lower band as indicated by braces denotes band shifts. Lane 1 is a competitive control where cold unlabeled probe was added in 100-fold excess. Results shown are representative of three independent experiments.

Because nuclear Nrf2 levels, in particular, are a direct reflection of ARE-mediated transcriptional activity, constitutive GCL gene expression by Nrf2 and other transcription factors binding to the ARE may decline with age. To examine whether this loss in constitutive nuclear Nrf2 levels translated into lower ARE binding, gel mobility shift assays were performed. As shown in Fig. 6.2B, we observed lower transcription factor binding to the consensus ARE sequence in nuclear extracts taken from old vs. young rats. Semiquantitative analysis of these EMSA experiments revealed a 40% loss in ARE-binding activity. These results are consistent with the 50% decline in nuclear Nrf2 levels as well as the observed loss in GCL subunit message levels with age.

To identify whether Nrf2 was, in part, responsible for the age-related attenuation in transcription factor binding to the ARE, supershift analysis was performed by using specific antibodies to Nrf2 (Fig. 6.2C). Results showed two distinct bands that were retarded in nuclear extracts from both young and old rats. Both of these bands were specific for Nrf2 as demonstrated by competition controls where the unlabeled ARE probe was added in 100-fold excess. These bands may reflect differences in heterodimer partners with Nrf2. Both of these shifted bands markedly declined with age, therefore demonstrating a distinct loss in Nrf2-dependent ARE binding activity.

Pharmacological Activation of Nrf2-Dependent ARE-Gene Transcription

Lower constitutive nuclear Nrf2 levels also suggest that normal signals to induce Nrf2 nuclear translocation may be altered with age. If true, disulfide chemopreventive agents, which readily induce Phase II enzyme response with respect to Nrf2, may not be beneficial to old rats. To test the degree of Nrf2 activation in old rats, we treated animals with LA, a disulfide compound known to activate Nrf2 (318, 319).

As shown in Fig. 6.3, hepatic nuclear Nrf2 levels in old rats treated with LA showed a marked increase over that seen in vehicle-treated controls. Age-related deficits in nuclear Nrf2 were largely abated within 12 h after LA treatment. This elevated level of nuclear Nrf2 was still evident 24 h after LA administration but declined sharply after 48 h. Thus, responsiveness of Nrf2 nuclear translocation when exposed to chemopreventive agents remains intact in the aging rat liver.

To further examine whether LA treatment caused increased transcription factor binding to the ARE, EMSA experiments were performed. As expected by the increase in nuclear levels of Nrf2, ARE transcription factor binding activity increased in response to LA as early as 12 h and remained elevated for up to 48 h (Fig. 6.3B). Supershift analysis showed that LA increased Nrf2 binding to the ARE vs. the vehicle control. A time course revealed that Nrf2 binding was maximal 24 h after LA treatment and declined after 48 h. Both of the bands associated with Nrf2 were similarly affected with LA (Fig. 6.3C). These results demonstrate that Nrf2 is responsive to exogenous activators in old rats.

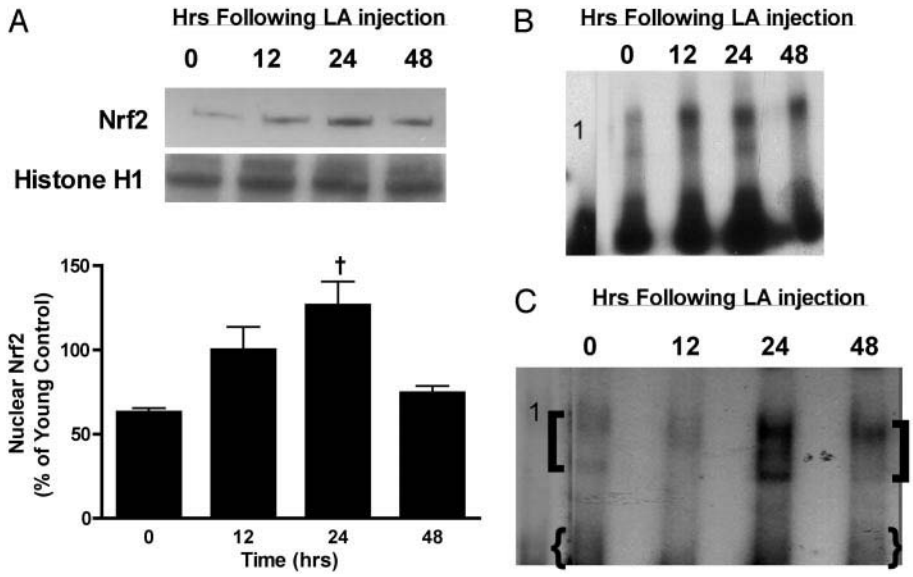

Figure 6.3 LA induces nuclear Nrf2 levels and increases its ARE binding activity. The time-dependent changes in nuclear Nrf2 levels after LA injection (40 mg/kg of body weight) in old rats were determined by Western blot analysis (A). The histone H1 normalized values are graphically represented and show an increase in nuclear Nrf2, with maximum induction seen within 24 h after LA injection (A). (B) EMSA analysis of nuclear extracts shows increased transcription factor binding to the ARE consensus sequence within 12 h, which was maintained for 48 h. Lane 1 is a competition with cold unlabeled probe. (C) Results from supershift assays, which indicate that LA increases Nrf2 binding to the ARE in a time-dependent manner and show maximal binding at 24 h after LA injection. Lane 1 shows a negative control using an antibody against the P65 subunit of NF-κB. Results are representative of three independent experiments. †, The group that is significantly (P <0.05) different from old control rats.

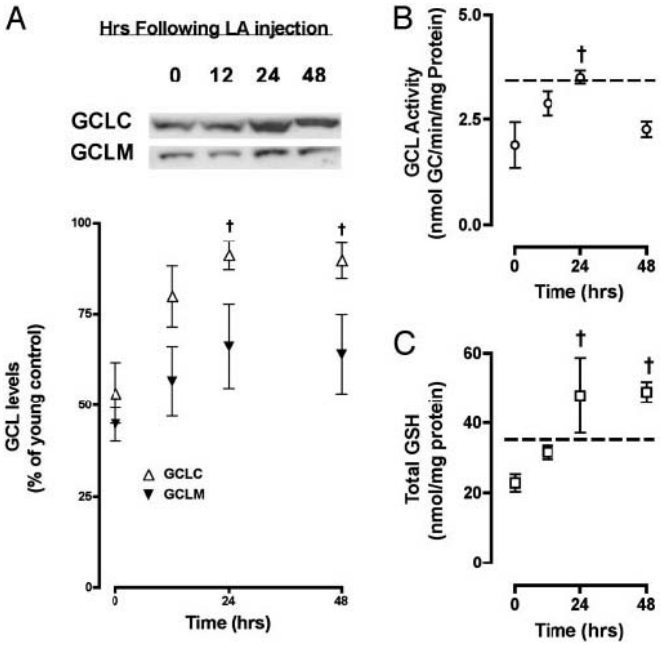

Figure 6.4 LA improves GSH synthetic capacity and hepatic GSH levels. Western analysis of GCL subunits was performed after LA injection at times indicated in A. Results show that GCLC levels in old rats were maximally increased in response to LA within 24 h (A). GCLM also exhibited a similar time-dependent increase, but the difference observed was not statistically significant (A). Paralleling the increase in GCL levels, enzyme activity increased by two-fold within 24 h after LA injection (B). The changes in GCL levels and enzyme activity resulted in an overall increase in hepatic GSH 24 h post LA injection (C). The dashed lines in B and C indicate mean experimental values seen in young animals. Results are expressed as the mean $\pm$ SEM. †, Groups that are significantly ($P \pm 0.05$) different from old controls.

To understand whether the LA-mediated increase in Nrf2 binding translated into elevated GCL levels, we measured GCLC and GCLM protein content in rats treated with LA or saline. LA treatment reversed the age-related decline in GCLC levels within 24 h (1.7-fold-increase; $P < 0.05$; Fig. 6.4A) after treatment. GCLM levels also increased; however, the heightened levels of this subunit did not reach statistical significance relative to vehicle controls (Fig. 6.4A).

As predicted by higher GCL protein content, hepatic GCL activity and GSH levels also significantly increased after LA treatment. Heightened GCL activity was observed over the 48-h time course and directly correlated with the LA-induced increase in GCLC and GCLM protein (Figure 6.4B). Concomitant with heightened GCL levels and activity, hepatic GSH levels also increased and were actually higher than that seen in young untreated animals 24 h after LA administration (Figure 6.4C). These results demonstrate that, just as in young animals, the nuclear translocation of Nrf2 efficiently elevates GSH synthesis in old rats.

6.5 Discussion

The present study demonstrates that one potential mechanism underlying the loss of GSH synthesis in old animals is a decline in Nrf2-mediated transcription of GCL proteins. More than 200 antioxidant and detoxication enzymes are regulated by the Keap1-Nrf2 pathway (320); thus, alterations in basal hepatic and nuclear Nrf2 levels and ARE binding activity are likely to have broad effects on cellular antioxidant and xenobiotic responses. In support of this notion, we also have preliminary evidence that NQO1 activity, a quintessential Phase II enzyme regulated by the ARE, declines with age (S.V.S., unpublished results). This finding raises the intriguing possibility of a global repression of Phase II response during aging.

Several lines of evidence [e.g., Nrf2-null mice, Nrf2 overexpression systems, and Nrf2-dominant negative mutants (146, 148, 314-316, 321, 322) implicate this transcription factor as a central transcriptional regulator of ARE-containing Phase II detoxication genes. For example, Nrf2-null mice share remarkable similarity to old animals, including loss of constitutive GCL levels and activity and a concomitant decline in GSH (202). The loss of stress tolerance capacity exhibited by Nrf2-null mice against toxicants, such as acetaminophen, mirrors a similar loss in aging rats (317). Thus, age-associated changes in GCL activity and in GSH levels may be a useful surrogate marker in understanding the mechanisms underlying transcriptional dysregulation of ARE-containing genes.

Like other Phase II genes, the enhancer regions of both GCLM and GCLC contain the ARE, which is most critical for basal and inducible expression of GCL (114, 117, 202, 323). Although the ARE seems to be the principal regulator of GCL expression, it is also noteworthy that its 5' promoter region also contains multiple binding sites for other transcription factors, such as NF-κB, SP-1, activator protein-1 and -2 (AP-1 and -2), and metal response (MRE) and antioxidant response element (74, 117, 324). Interestingly, a study using rats reported that basal and inducible GCL transcription depends on AP-1 rather than the ARE, as reported in humans and mice (117). Although the reasons for this discrepancy are not clear, one possibility is that the ARE sequence that is functionally important for GCL regulation resides in a region not examined in that study (117). Although this finding raises the possibility of AP-1 involvement in regulating rat GCL, the results from the current study, as well as those from a previous report, support at least a role for Nrf2 in regulating GCL expression, even in rats (325). Further studies are needed to delineate how the ARE and other responsive elements interact to govern GCL transcription and the role of other transcription factors in the age-related loss of GSH biosynthesis.

The control of Nrf2-dependent transcription can be achieved at multiple levels: (*i*) by Nrf2 interactions with Kelch-associated protein 1 (Keap1), a cytosolic repressor protein, and/or (*ii*) by Nrf2 interactions with other bZip transcription factors. Nrf2 bound to Keap1 has a short half-life (~20 min) and is rapidly degraded by ubiquitin-26S proteosomes (152). Keap1 [inhibitor of Nrf2 (INrf2) in rats] has abundant free cysteine residues (25 cysteines), which makes it an ideal redox-sensing partner (326).

A recent study reported that the specific modification of Cys-151 leads to the dissociation of Nrf2 from Keap1 (326), which prevents Nrf2 degradation and allows its nuclear translocation. The paradoxical decline in nuclear Nrf2 levels despite an increased age-associated pro-oxidant cellular milieu, suggests that there may be potential alterations in the redox-sensing capacity of Keap1. To partially address this issue, we treated old rats with LA, a disulfide compound known to activate Phase II gene transcription (318, 319). We found that LA potently increases hepatic nuclear Nrf2 levels in a time-dependent manner. These results demonstrate that the pathways leading to Nrf2 activation remain intact in old animals.

Aside from changes in Nrf2 and Keap1 interactions, other factors may be responsible for the observed decline in Nrf2 levels. Nrf2 gene expression is itself governed by the ARE (327); therefore, the intriguing possibility exists that the dysregulation that alters ARE-mediated gene transcription also detrimentally represses Nrf2 transcription. Corroborating this hypothesis, we also observed an age-dependent loss of total cellular Nrf2 levels. It will be important to examine the exact nature and mechanism(s) underlying the age-related decline of Nrf2.

A further complicating factor in characterizing the age-related loss of ARE-dependent gene transcription is the influence of different transcription partners to Nrf2. Nrf2 forms heterodimers with other bZip proteins, including the Jun-Fos family, Fra, small Maf, and ATF4 proteins (114, 145, 146, 328). Depending on the partnering factor, Nrf2-dependent gene expression can be modulated, either positively or

negatively, to a significant degree. It is not known whether relative proportions of these partner proteins are altered in the aging rat liver. It will be important to analyze potential age-related changes in these factors, particularly, the Jun-Fos family of proteins and their effect on ARE-mediated gene transcription.

In summary, the evidence presented in this paper shows a reduced Nrf2-mediated gene expression in the aging rat liver. Remarkably, activation of Nrf2 can be achieved by treating old rats with LA. In addition to contributing to our understanding of the aging process, these findings suggest new strategies to improve stress resistance in the elderly. Related chemo preventive strategies have garnered much interest in cancer research but, to our knowledge, have heretofore not been advocated as a possible means to improve the morbidity and mortality associated with aging. The results presented herein strongly suggest that further research, in this regard, is warranted (34, 329).

Chapter 7

General Conclusion

7.1 General Conclusion

Aging is associated with an increased risk for morbidity and mortality, making the elderly the most vulnerable group to all kinds of degenerative diseases (330, 331). According to a recent survey by the U.S. Department of Health and Human Services, 40% of Americans over age 65 exhibit at least one chronic disease, disability, or other functional deficit that limits normal daily activity (41, 330, 332, 333). This number rises to over 90% of respondents that are 85 and older (3). Considerable medical advances have been made in mitigating symptoms of cardiovascular diseases, neurodegeneration, cancer, diabetes and loss of kidney function – all common problems afflicting the aged population. The net result is management of these diseases through an extensive array of drug regimens in the elderly and an extension in the mean lifespan. On an average, people over the age of 65 take one to four drugs on a daily basis (41). Hence, it is no surprise that 75% of the healthcare cost in the U.S. is spent on the elder age group (330).

However, a common side-effect that is frequently overlooked is the inability to handle multiple pharmacological drugs with increasing age (41). Stress-response and drug detoxification capacity drastically decline in older individuals (2, 4, 290). Concomitantly, antioxidant defenses do not keep pace with the age-induced susceptibility to stress-response (334, 335). This indicates that though the elderly are living longer, they are not really living well. It also points for a need to boost overall endogenous defenses, not just treat symptoms after manifestation of disease. A key facet of improving elder health would involve a two-pronged plan: 1) identify the

precise reasons why a highly inducible antioxidant response system is dysfunctional with age, and 2) develop strategems to safely maintain stress response mechanisms in older individuals. Such an approach has been conspicuously lacking in the field of aging research until recently, where most of the work has been characterized by phenomenology and not a molecular- or therapeutic-based approach. Because of this precise reason, this dissertation project has been guided by the above-mentioned two goals.

As described in the previous chapters, a consistent underlying symptom of aging is a decline in the abundant low molecular weight antioxidant, GSH. The phenomenon of age-related GSH loss and the multiple pathways of synthesis, efflux, and feedback regulation contributing to its availability have been described extensively in literature (35, 50, 93). However, no significant attempt has been made to date in the direction of pinpointing a fundamental molecular mechanism leading to inadequate GSH levels and consequently, reduced stress response with age.

In this context, we directly looked at the transcriptional regulation responsible for lower GSH levels in the elderly. Our finding that GSH synthesis is the limiting factor during aging (see chapter 6) points to a common fundamental flaw in highly inducible defense systems in aging individuals. Furthermore, the discovery that a lesion in the Nrf2-ARE system is central to the age-related loss in GSH synthesis represents an important advance in the field of gerontology. This is especially because the Nrf2-ARE regulon controls the induction of over 100 antioxidant defense and

detoxification genes. A majority of genes regulated by this system have been directly implicated to play a role in the development of degenerative diseases; for example, NQO1 in cancer, HO-1 in neurodegeneration, GST2A in kidney failure, ferritin in diseases of iron accumulation: the list is endless. Thus this transcriptional network is at the core of all the diseases and the related morbidities that the elderly are prone to.

Our work has shown that the amount of Nrf2 available in the nucleus to carry out its transcriptional function significantly declines in the aging liver (see chapter 4). This depletion of Nrf2 induces a previously uncharacterized partial chromatin silencing at the active ARE element in the *Gclc* promoter. The transcription factor profile consisting of the repressor Bach1 suggests that chromatin is now tightly wound and recalcitrant to any type of transcriptional induction. As a consequence, Nrf2 shifts from this inactivated promoter to another site resembling the ARE to direct transcription in aging cells. This promoter shifting by Nrf2 reveals a striking feature common to loss of function in many parameters during aging: there is a considerable effort to overcome the lesion in fundamental processes by induction of a compensatory mechanism.

Similar to the loss of GSH synthetic enzymes with age, we have also identified age-related declines in a number of key phase II detoxification and antioxidant enzymes. These include NQO1, GST2A, HO-1, and the cysteine X_C transporter (chapter 3 and data not shown). Hence the elucidation of the transcriptional mechanism of GSH synthesis raises the intriguing possibility of a similar mechanism

involved in the repression of phase II genes during aging. Accordingly, the most tantalizing direction for this work would be to explore whether there exist similar alternative "ARE-like" elements in the promoter regions of other phase II genes that are downregulated with age. Due to the sequencing of the human, mouse and rat genome and the availability of computational ARE-identification programs, such a venture would be relatively easy and extremely informational. This can be followed by assessing whether these sequences are unmasked for Nrf2 binding using techniques like chromatin immunoprecipitation of Nrf2 followed by a microarray (ChIP-on-chip) to identify target genes (336, 337).

A lucrative causal factor for the age-related silencing of the prototypical ARE site is the transcriptional factor Bach1. As outlined before, the presence of Bach1 likely makes the ARE resistant to activation by Nrf2 (chapter 4). In this regard, Bach1 can be effectively supressed by heme in the context of the HO-1 gene to permit ARE-mediated transcription (338). Thus, Bach1 is potentially an attractive target for mitigation of Nrf2-ARE repression during aging. Taken together, research presented in this dissertation has opened the door to many future studies exploring molecular mechanisms of lost stress response during aging.

Keeping in accordance with the second objective of this dissertation to identify safe and natural therapeutic interventions to maintain stress response systems in the elderly, we tested the micronutrient dithiol LA in maintenance of GSH. LA effectively reverses the age-related decline in GSH levels (chapters 5 and 6). While similar

studies from other groups might suggest that this is due to the antioxidant-sparing capacity of LA, the dynamics of LA tenure in tissues does not support this notion (178, 182-188). Results from animal and human studies show that while LA is readily bioavailable from the diet and transiently increases in most tissues following ingestion, non-protein bound LA neither accumulates or persists at high levels in tissues (308-310, 339, 340). This points to a different mechanism of LA-mediated GSH induction.

In fact, we showed that LA reverses the age-associated loss of GSH by maintaining the expression and levels of GSH synthetic enzymes that otherwise decline (chapter 6). LA also induces nuclear localization of Nrf2 and Nrf2-ARE binding that is inhibited with age. Surprisingly, the target of LA action is not the normal ARE element, but the age-specific alternate ARE site (chapter 5). This finding again underscores the observation that the chromatin configuration of the normal ARE element is severely hampered with age. Additional support of this idea also comes from the data that LA treatment does not alter the levels of Bach1 or other Nrf2 partner proteins at the ARE promoters. Thus, LA solely targets Nrf2 nuclear levels and subsequent ARE binding.

What, then, is the mechanism, whereby LA induces the nuclear translocation of Nrf2? While this dissertation did not specifically investigate this question, certain speculations can be made based on the action of LA at the transcriptional level. Because of the high electron density of the dithiolane ring, nonprotein-bound LA

could theoretically react with the redox sensitive cysteine groups of Keap1, catalyzing Nrf2 release or its slower degradation (305). Either scenario would result in a higher Nrf2 nuclear accumulation. This concept is supported by similar mechanisms shown for other known Keap1 thiol modifiers (e.g. sulforaphane and the dithiol thiones) (150, 326, 341, 342). Alternatively, LA can also act as a mild oxidant, which would again elicit a moderate modification of Keap1 thiol redox state. With this similar rationale, many cell signaling pathways, which are known to be involved in Nrf2 phosphorylation (e.g. Akt, PKC, and MAP kinase pathways) and attendant nuclear translocation are induced under mild oxidative insult (305, 308).

Thus, this dissertation forms the basis for future studies examining the universality of the 'LA effect' on Nrf2-ARE regulated genes. If LA does indeed affect other phase II defenses through a transcriptional mechanism similar to that outlined above, new therapeutic possibilities would open up for its use in maintaining elder health. In this context, it is necessary to make a distinction between LA and other common imducers of the Nrf2-ARE system. The transient nature of its accumulation may actually make LA a better Nrf2-activating agent versus stronger compounds that elicit a sustained Phase II induction. Such chronic Phase II activators may be detrimental, leading to less cell turnover and increased risk for mutagenesis. In addition, relevant to its role as an age-specific hormetic agent, LA merely maintains Nrf2 homeostasis during aging, but does not increase Nrf2-ARE binding indiscriminately. Based on these observations, we have fulfilled the dual goals of identifying LA as a potential 'age-essential' micronutrient as well as utilized it as a

'nutritive key' to unlock the transcriptional mechanism of GSH synthesis during aging.

Other than identifying the age-related alterations in transcriptional regulation of GSH and its remediation by LA, this dissertation has utilized novel research techniques previously limiting aging research. A case in point is the establishment of conditions whereby freshly isolated hepatocytes from young and old rats can be placed in culture and their respective aging phenotypes maintained (chapter 3). The development of such a primary culture model is not trivial for gerontology. Culture models so routinely used in many other disciplines have been lacking for aging research. Thus, we have overcome a significant technical obstacle that has prevented understanding of molecular and cellular lesions involved in the age-related decline in stress resistance.

In summary, this dissertation has fulfilled the dual goals of identifying a fundamental mechanism for the age-related loss of stress response systems as well as identifying a potential nutritional intervention to maintain these systems in the elderly.

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APPENDIX

List of Publications

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