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Glutathione maintenance mitigates age-related susceptibility to redox cycling agents

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ABSTRACT

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

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1. Introduction

A major hallmark of aging, and a key driver for the onset of age-related pathophysiologies across multiple species, including primates, is the disruption of cellular redox homeostatic mechanisms that protect against a variety of environmental, oxidative, pathological, and toxicological insults [1–6]. Nrf2-dependent phase II detoxification mechanisms in particular, tend to decline with age [7–12]. The age-related decrease in these detoxification pathways and ensuing increase of reactive oxygen/nitrogen species (ROS/

RNS) is well established and is causally linked to various pathologies such as cardiovascular and neurodegenerative diseases, cancer and diabetes [13–22]. The mechanisms associated with this loss are poorly understood; however, we have found that hepatic Nrf2 protein synthesis declines with aging and that phase II detoxification gene expression is limited [7,23,24]. However, despite the age-related decline in basal expression of Nrf2 regulated detoxification enzymes, it remains unknown whether this loss magnifies the toxicological exposure effect of ROS/RNS and xenobiotics detoxified through these pathways. Of particular interest to this work are the age-associated changes to resilience against acute exposure to a redox cycling challenge.

Redox cycling compounds are prooxidant catalysts, which facilitate the transfer of electrons onto oxygen to produce reactive oxygen species (ROS) [25,26]. These compounds are highly abundant as substituents in xenobiotic compounds (e.g. redox active metals and pesticides) [27–31], redox active pharmacophores (e.g. anesthetics) [32], and especially, their use in pharmacotherapeutic drugs (e.g. menadione, anthracycline, adriamycin, and doxorubicin) [33–37]. A decreased capacity to detoxify redox

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

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cycling agents in the liver could potentially increase vulnerability to xenobiotic exposures, as well as limiting some medical treatment options such as antibiotics and anti-cancer chemotherapeutics [38–41]. This is of particular importance as cancer incidence increases exponentially with age [42]. Thus, it is important to determine whether there is an age-related decline in detoxification of redox cycling compounds and if so, which of these types of drugs, toxins, or environmental xenobiotics have a heightened toxicity profile.

In order to test our hypothesis that there is an age-related decline in resilience to redox cycling compounds in the liver, we employed an acute menadione challenge. Menadione, a derivative of vitamin K and a redox cycling agent, and its mechanism of action is well characterized. Herein, we show that hepatocytes from aged rats are acutely more susceptible to menadione insult. Moreover, while certain detoxification enzymes involved in menadione metabolism actually increase with age, the observed age-associated vulnerability to menadione appears to stem from a marked attenuation of Nrf2-regulated GSH-dependent detoxification pathways.

2. Materials and methods

2.1. Reagents

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

2.2. Animals

Both young and old male F344 rats were from the National Institute on Aging animal colonies. The rats were housed in the Linus Pauling Institute animal facility and allowed to acclimatize for at least 1 week prior to any experimentation. Animals were maintained on a 12 h light cycle (7 a.m.–7 p.m.) and fed standard chow ad libitum. All animal work was approved and in accordance to IACUC guidelines (Assurance Number: A3229-01). The AAALAC-accredited Laboratory Animal Resources Center (LARC) provided management and veterinary care.

2.3. Hepatocyte isolation

Hepatocyte isolation was performed as described previously [43]. Briefly, after animal sacrifice via AALAC-approved protocols, the liver was perfused via a cannula in the portal vein with Hank's balanced salt solution, pH 7.4. Following removal of blood, liver cells were disassociated using collagenase solution (1 mg/mL). The resultant cell suspension was filtered through sterile gauze to remove connective tissue and debris. Parenchymal cells were isolated using gravity filtration and washed three times with Krebs-Henseleit solution, pH 7.4. Cells were resuspended in Krebs-Henseleit solution and placed in a round bottom flask and rotated at room temperature for 1 h before cell count and viability were assessed using trypan blue exclusion.

2.4. Cell and tissue lysates

For whole cell lysates, suspended cells were harvested by centrifugation at $100 \times g$, washed in Krebs-Henseleit solution, pH

7.4, and sonicated in lysis buffer (50 mM Tris, pH 7.5, containing 1% NP-40 (v/v), 100 mM NaCl, 2 mM EDTA, 2 mM sodium ortho-vanadate) with added protease inhibitors. For tissue, lysates were obtained as previously described by Siegel et al. [44]. Briefly, tissue was homogenized using a dounce homogenizer in RIPA buffer with a volume to weight ratio of 5:1. The homogenate was sonicated 3 times and centrifuged for 15 min at $10,000 \times g$ centrifugation (4 °C) before supernatants were collected for assays. For the NQO1 assay, supernatants from tissue lysates were subjected to an additional ultracentrifugation step ($30,000 \times g$ for 1 h at 4 °C). Protein concentrations of samples were determined either by the Bradford Assay (Cat# 500-0006, BioRad) or Pierce 660 nm assay (Cat# 22660, Thermo Scientific).

2.5. Assessment of menadione toxicity

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

2.6. NQO1 activity assay

NQO1 activity of samples was assayed as described previously by Siegel et al. [44]. Briefly, tissues from young and old animals were prepared as described above before being assayed for the NAD(P)H-dependent reduction of DCPIP by NQO1 in the presence and absence of dicumarol (a reversible NQO1-specific inhibitor). DCPIP reduction was assayed using a DU800 spectrophotometer at 600 nm over 1 min, and NQO1 activity was measured as the dicumarol inhibitable portion of the reduction. Final concentrations of reagents in reaction solution were 0.2 mM NAD(P)H and 40 μM DCPIP with and without 20 μM dicumarol.

2.7. Malondialdehyde quantification

Measurement of the lipid peroxidation product, malonyldialdehyde (MDA), was performed as previously described by Wong et al. [45] and modified by Sommerburg et al. [46]. Briefly, 200 μL of suspended cells (4×10^6 cells/mL) was mixed with 750 μL of 440 mM phosphoric acid, 250 μL of 42 mM thiobarbituric acid (TBA), and 300 μL of water prior to being boiled for 1 h. The reaction was quenched by placing samples into an ice bath. Samples then had an equal volume (1.5 mL) of 1 M NaOH added before being centrifuged at $16,000 \times g$ for 5 min at 10 °C. Malondialdehyde was separated from other metabolites by HPLC using a Luna C18(2) Phenomenex #00G-4252-E0 column in isocratic mode (25 mM potassium phosphate buffer, pH 6.5/methanol [50:50] as eluents) and detected by fluorescence (excitation, 532 nm; emission, 553 nm). Malondialdehyde was quantified relative to a tetramethoxypropane (TMP) standard curve.

2.8. Immunoblotting

Lysates were prepared as described above, sonicated, and proteins were solubilized for PAGE in Laemmli loading buffer containing SDS. Samples were heat-denatured for 5 min at 100 °C. Normalized amounts of protein (30 μg/lane) were run on SDS-PAGE and transferred to PVDF membranes with a semi-dry blotter. Membranes were blocked in PBS containing 1% Tween-20 with

either 5% nonfat dry milk or 3% BSA, incubated with primary antibodies for 2 h at room temperature, washed, and incubated with secondary antibodies for 1 h at room temperature, washed, incubated with chemiluminescence reagents, exposed to film, and developed. Images shown were cropped for size/space considerations. Antibodies made to the following proteins were used: GPX4 (Protein Tech - Cat# 14432-1-AP), NQO1 (Abcam - Cat# ab2346), Cytochrome P450 Reductase (CPR, EC 1.6.2.4; Abcam - Cat# 13513), Lactate Dehydrogenase A (LDHA; Cell Signaling Technology - Cat# 2012) and Actin (Sigma-Aldrich - Cat# A5044). Blots were densitometrically analyzed with ImageJ software from NIH.

2.9. Glutathione analysis

Glutathione (GSH) content of suspended cells was determined according to the methods of Fariss and Reed [47] as modified by Smith et al. [7]. Briefly, suspensions were homogenized in an equal volume of 10% (w/v) perchloric acid (PCA) containing 10 mM EDTA. After deproteinization, 200 μ L of the supernatant containing internal standard (γ -glutamyl glutamate) was mixed with 50 μ L iodoacetic acid (100 mM dissolved in 0.2 mM *m*-cresol purple) and the pH was adjusted to 10 by using KOH–KHCO₃ buffer (2 M KOH:2.4 M KHCO₃). Samples were placed in the dark, at room temperature for 1 h. The resulting *S*-carboxymethyl derivatives were subjected to further chemical modification by addition of an equal volume of 1-fluoro-2,4-dinitrobenzene (1% v/v in absolute ethanol). The solution was incubated overnight in the dark and at room temperature. Samples (75 μ L) were separated by HPLC using a Thermo Scientific (Eugene, OR) APS-2 Hypersil column and detected on a Shimadzu (Kyoto, Japan) SPD-10AVP UV–Vis spectrophotometer with the absorbance set at 365 nm. Quantitation was obtained by integration relative to GSH and GSSG standards.

2.10. Statistical analysis

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

3. Results

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

To establish the relative age-associated susceptibility to menadione challenge, aliquots of freshly isolated hepatocytes from young and old rats were incubated with increasing concentrations of menadione, and viability was assessed after 2 h of challenge. Results in Fig. 1 show that regardless of age, increasing concentrations of menadione corresponded with heightened indices of cell damage and loss of viability. For hepatocytes from young rats, the lowest concentration of menadione employed (100 μ M) resulted in no changes in toxicity versus vehicle control; in contrast, hepatocytes isolated from old rats lacked any resilience to menadione, demonstrating viability loss at all concentrations tested. The LC₅₀ for menadione was calculated to be 405 μ M and 275 μ M for young and old rat hepatocytes, respectively (Fig. 1). These results indicate a significantly higher susceptibility to menadione with age ($p < 0.05$, $N=3$), and suggest that basal cellular defenses to this redox cycling agent are compromised.

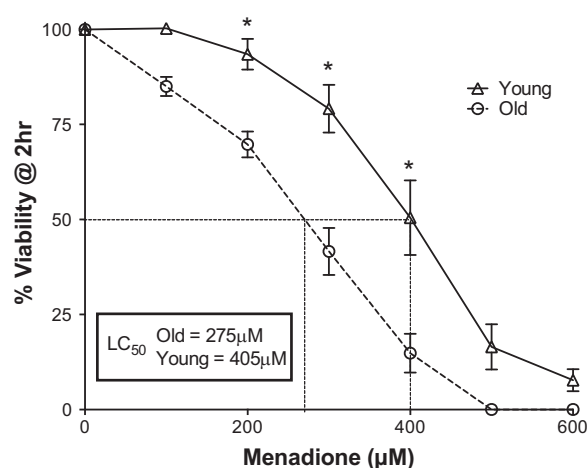


Fig. 1. Acute menadione toxicity is enhanced with age. Isolated hepatocytes from young and old rats were treated with increasing amounts of menadione (0–600 μ M). Viability was determined by trypan blue exclusion 2 h after exposure. $N=3$, * $p < 0.05$.

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

Considering the mechanisms associated with its detoxification (Fig. 2), heightened age-dependent vulnerability to menadione may stem from altered activities of one or more antioxidant or phase II detoxification enzymes. As almost all of these enzymes are primarily regulated by Nrf2 [48–54], we sought to define potential lesions in menadione detoxification, which might serve as the basis for its enhanced toxicity with age.

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

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

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

Menadione and the reactive oxygen species initiated by its redox cycling are primarily detoxified by GSH-dependent mechanisms (cf. Fig. 2), which we previously reported to decline significantly with age [7]. In order to determine whether hepatic GSH levels become limiting under menadione insult, hepatocytes from young and old animals were treated with menadione at approximately the LC₅₀ for old rats (300 μ M). Results show that

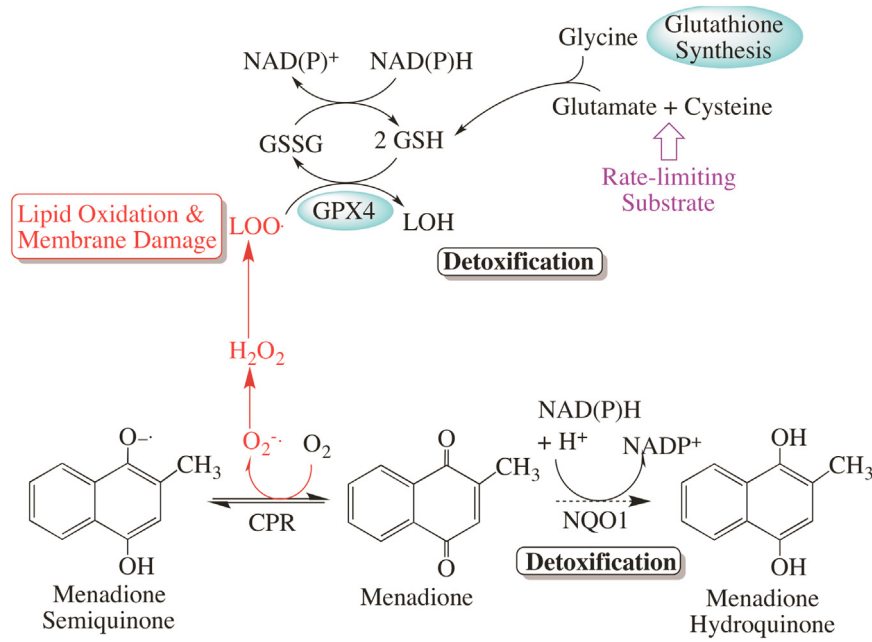


Fig. 2. Menadione detoxification scheme. Menadione is a potent redox cycling agent which cycles between the quinone and semi-quinone states, producing superoxide, which may eventually lead to oxidative and nitrosative damage. NQO1 detoxifies the menadione quinone by reducing it to the hydroquinone, which is then eliminated. The major resultant oxidative damage from redox cycling is lipid peroxidation (LOO \cdot), which is primarily detoxified by the glutathione-dependent GPX4 enzyme.

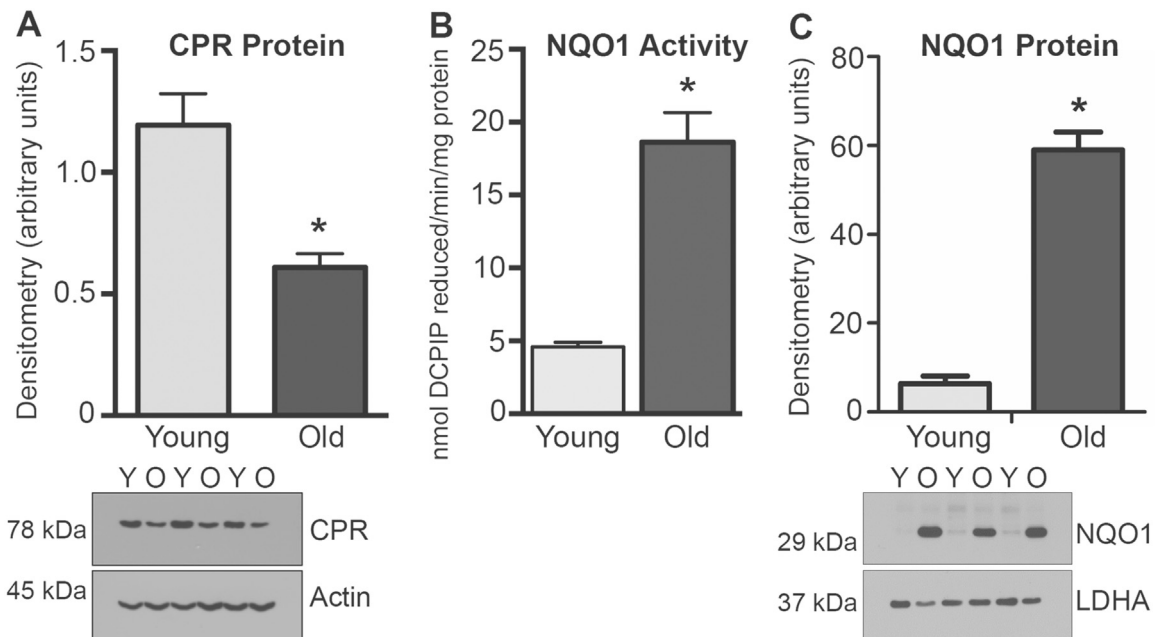


Fig. 3. CPR protein levels decrease while NQO1 protein levels and activity increase with age. CPR protein levels (A), and NQO1 activity (B) and protein levels (C) in liver tissue homogenates from young (Y) and old (O) animals were evaluated. $N=3$ for A, B and C, * $p=0.0137$, 0.0185 and 0.0019 respectively.

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

To further determine age-associated differences in GSH-dependent detoxification of menadione, we examined hepatic concentrations of glutathione peroxidase 4 (GPX4). This enzyme is a primary protectant against phospholipid hydroperoxides, and is also known to play a significant role in protection against redox cycling [55,56]. Western blot analysis revealed that GPX4 protein levels were significantly lower in old than in young hepatocytes (Fig. 5A; 70%, $p=0.0043$, $N=3$), which is consistent with reports of age-related loss in GPX4 levels in multiple tissues, including the liver [57,58].

Because GPX4 chiefly protects against lipid peroxidation, we measured hepatic levels of malondialdehyde (MDA), a biomarker

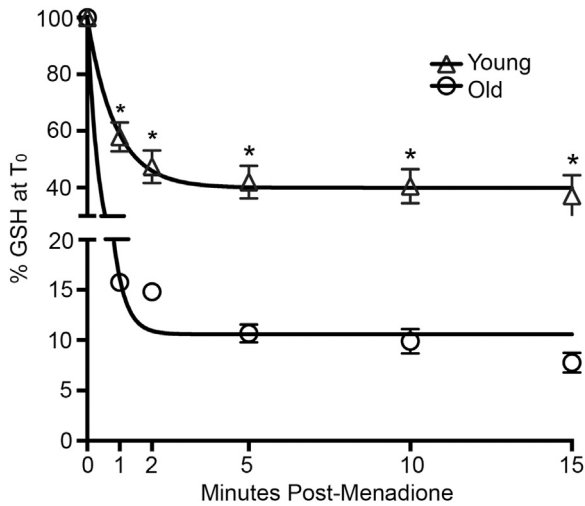


Fig. 4. GSH loss during menadione treatment is accelerated with age. Hepatocytes from young and old rats were treated with 300 μ M menadione over a 15 min time course and assayed for GSH content. N=3, * $p \leq 0.01$.

of the oxidation of polyunsaturated lipids, through its derivatization with thiobarbituric acid (TBA). Because TBA derivatization may overestimate lipid peroxidation levels, we employed a well-characterized HPLC method to minimize ex vivo artifacts so that relative differences in lipid peroxidation with age and menadione could be discerned. [59] It is interesting to note that, without menadione, no age-associated differences in MDA levels were observed, indicating that basal lipid oxidation and its detoxification are maintained despite the age-related loss of GPX4 (Fig. 5B). However, exposure to menadione at the approximate LC_{50} for old rat hepatocytes (300 μ M), resulted in a rapid appearance of MDA in cells from both age groups (Fig. 5C). Hepatocytes from aged rats were demonstrably more vulnerable to menadione-mediated lipid peroxidation. Initial increases in MDA levels for cells from both young and old animals was observed, followed by a brief stabilization of MDA levels between the 2 and 5 min time points. After this, MDA levels in cells from young animals returned to baseline. In contrast, hepatocytes from old rats displayed a rapid increase in MDA levels after the 2 through 5-min stabilization period. After 15 min, MDA levels were 147.4 ± 13.8 and 274.2 ± 35.1 pmol/mg protein in liver cells from young and old animals, respectively. Moreover, lipid peroxidation levels in hepatocytes from young animals never increased by more than 5%, whereas the extent of

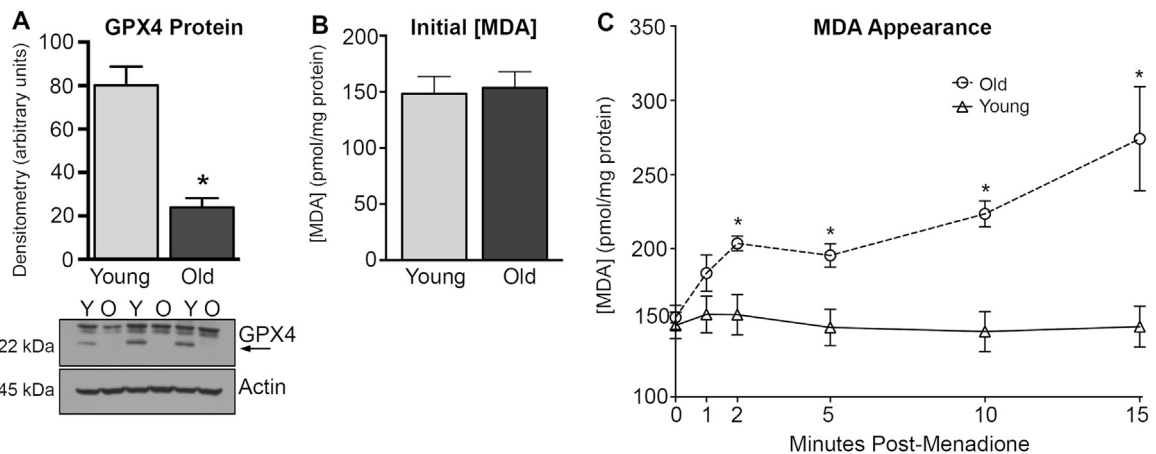


Fig. 5. GPX4 protein levels are significantly reduced with age which correlates with an increased appearance of menadione-induced MDA. Hepatocytes from young (Y) and old (O) animals were evaluated for GPX4 protein levels by western blot (A). Basal MDA content (B) and MDA accumulation (C) were assayed after menadione treatment. N=3 for A, B and C, * $p=0.0043$, > 0.05 , and < 0.05 for A, B, and C respectively.

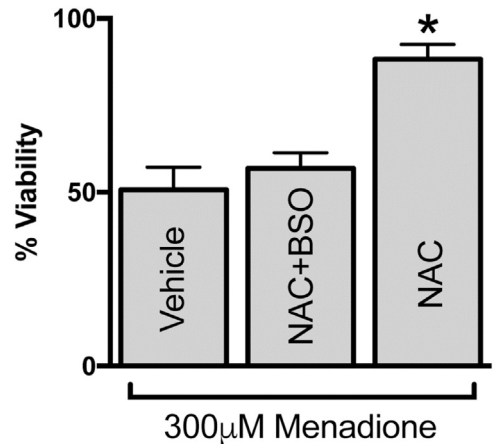


Fig. 6. Pretreating cells with NAC prior to menadione exposure improves resistance. Hepatocytes from old animals were pretreated with vehicle (water), 400 μ M NAC, or 400 μ M NAC & 400 μ M BSO for 1 h prior to addition of 300 μ M menadione. Viability was determined 2 h later. N=5, * $p=0.003$ versus vehicle.

lipid oxidation in hepatocytes isolated from old rats were 78% higher and still rising 15 min after menadione challenge. The divergence between young and old is striking and aligns with loss of GPX4, as well as the appearance of cellular damage and loss of viability.

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

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

4. Discussion

The aging process leads to disparate changes in hepatic detoxification capacity. For example, many phase I enzymes are unaffected by age, while phase II detoxification processes are more variable [60–62]. Our data exemplifies this with NQO1 levels and activity increasing significantly in aging hepatocytes, but GPX4 appreciably declines. Therefore, while a general age-related loss in basal detoxification capacity is apparent, it is not clear if this decline is at least partly compensatory, or leads to vulnerability to certain classes of xenobiotic compounds. Our present study is important as our data supports the concept that, at least for redox cycling compounds, (e.g. menadione) there is a profound susceptibility with age.

The current study focused on susceptibility to acute menadione insult using freshly isolated hepatocytes from young and old rats. This cell model is appropriate for understanding aging differences in detoxification because these cells retain their aging phenotype with respect to stress resistance. It also allows for a direct assessment of potential age-induced differences in hepatic detoxification capacity at the time of animal sacrifice [43]. Using this hepatocyte model, we previously showed that steady-state Nrf2 levels significantly decline both in hepatocytes and in the aging rat liver [7,23,24].

We used menadione in the current study because most of the enzymes involved in its detoxification are Nrf2-dependent. For example, NQO1 maintains menadione in its fully reduced hydroquinone state [25,26,63] prior to its further metabolism and removal from the cell (cf. Fig. 2). Additionally, because menadione is a potent redox cycling agent, its mode of acute toxicity results from a cascade of redox cycling, lipid peroxidation, and membrane damage [26,64–67]. As such, we chose to examine GSH and GPX4, the principal glutathione peroxidase that is localized to membranes, as the primary players in preventing cell damage from acute menadione exposure [68–71]. While GPX1 also plays a major role in peroxide detoxification, it is localized to the cytosol and thus is not the initial detoxifier of menadione-induced lipid peroxidation. Thus, xenobiotic disposition and the extent of acute oxidative damage ensuing from menadione exposure are largely dependent on the hepatocellular activity of NQO1, GSH, and GPX4—all of which are Nrf2-regulated mechanisms.

Despite this common tie to Nrf2 associated gene transcription, we found that aging does not uniformly affect all of the enzymatic components involved in menadione detoxification. For example, we observed that hepatic levels of NQO1 and its activity significantly increase with age, while CPR activity is diminished. Overall, these results could be interpreted as an attempt by the hepatocyte to limit levels of the semiquinone species, thereby lowering the rate of redox cycling. However, this protective adaptation against menadione insult in the aging rat liver is counterbalanced by the significant loss of GSH levels and GSH-dependent detoxification enzymes (e.g. GPX4). The reason(s) why certain Nrf2-dependent genes are affected with age while other genes do not change or decline are not presently known. With respect to NQO1, it has been reported that with age, NQO1 levels and/or activity decline [72], increase [73], or remain similar to that seen in young [74]. These results may stem from the varied methods employed to monitor NQO1. We used the reduction of DCPIP as first developed by Ernster et al. [75] and recently optimized by Ross et al. [44]. This method has the benefit of highly purifying the cytosolic fraction through ultracentrifugation and does not assay the reduction of menadione-cytochrome c, which has confounding NQO2 enzymatic activity. Additionally, the NQO1 promoter region contains an embedded TRE (TPA-response element) within the ARE enhancer [76,77] and demonstrates higher recruitment and binding efficiency for Nrf2 both for basal and

induced transcription [78,79]. These factors may account for the increase we observed in spite of Nrf2 decline. Regardless of the precise mechanism, it is clear that NQO1 is not involved in the age-related enhanced vulnerability to menadione that we observed in this study.

It is equally clear that GSH-dependent defenses are compromised in hepatocytes from aging rats. We and others have previously shown that hepatic GSH declines with age [7,80–84]; however, GSH remains at millimolar levels even in very old animals. Thus, it was not known prior to the present study whether the liver is more susceptible to menadione insult that would result from attenuated GSH concentrations. Our results show that menadione causes a rapid loss of GSH in hepatocytes from both young and old rats, but the rate and degree of GSH loss is more extensive in older animals. This lack of capacity to maintain cellular GSH appears to be the most important factor leading to lost resiliency against menadione challenge with age. This concept is buttressed by our results using NAC and NAC+BSO to modulate GSH prior to menadione insult. In particular, NAC, which supplies L-cysteine for GSH synthesis [85–87], essentially abrogates the increased vulnerability to menadione in aged rat hepatocytes. Taken together, the inability to supply GSH for detoxification, along with diminished GPX4, potentially sets the stage for the aging cell to be susceptible to a variety of toxins, oxidants, and environmental mutagens.

An additional aspect revealed in this study is that GSH levels in young rat hepatocytes never decreased below ~35% of initial levels versus the 90% lost by old. This suggests that when the menadione-mediated oxidation crosses a critical threshold of GSH, cellular toxicity rapidly ensues. Because mitochondria contain a separate GSH pool that approximates this threshold level, it is enticing to suggest that maintenance of mitochondrial GSH is particularly important to resist menadione insult. In this regard, loss of mitochondrial GSH has been demonstrated to cause a decline in mitochondrial membrane potential [66,88–91], inducing calcium overload and initiating necro-apoptotic pathways leading to cell death [92–96]. We are currently investigating the role of mitochondrial GSH in sensitizing the cell toward oxidative insult in aging.

Finally, the results presented in this study identify a specific cellular target to potentially improve detoxification and xenobiotic metabolism. There is a significant clinical history of using NAC to limit toxicity from acute exposure to drugs and toxins [97–99]. Our data suggest that a similar preventative strategy of NAC administration may be warranted to increase resistance to xenobiotic and drug toxicity in older adults. GSH is a principal detoxicant for environmental xenobiotics, pharmaceuticals, air pollutants, and heavy metals. Providing NAC to increase substrate supply of cysteine may circumvent the age-related decline in GSH synthetic enzymes that attenuation of Nrf2 engenders. Thus, using NAC as a prophylactic instead of an intervention may allow GSH levels to be maintained for detoxification in older adults.

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