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Research paper

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 LC_{50} for each age group was established, which showed that aging resulted in a nearly 2-fold increase in susceptibility to menadione (LC_{50} for young: 405 μM; LC_{50} 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.

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 pharmaco chemotherapeutic 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; DCPIP, Dichlorophenolindophenol; GPX4, Glutathione Peroxidase 4; LDHA, Lactate dehydrogenase A; LOO, Lipid peroxide; MDA, Malonyldialdehyde; NQO1, NAD(P)H:quinone oxido-reductase 1; NQO2, NAD(P)H:quinone oxido-reductase 2; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; RIPA, Radioimmunoprecipitation assay; TRE, TPA-Response Element

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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 Kreb-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 Kreb-Henseleit solution, pH 7.4, and rotated on a MACSMIX (Miltenyi Biotec) rotator placed in a cell culture incubator (5% CO_2 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