Elevated Glutathione Is Not Sufficient to Protect against Doxorubicin-Induced Nuclear Damage in Heart in Multidrug Resistance–Associated Protein 1 (Mrp1/Abcc1) Null Mice^S

Jun Deng,¹ Donna Coy,² Wei Zhang, Manjula Sunkara, Andrew J. Morris, Chi Wang, Luksana Chaiswing, Daret St. Clair, Mary Vore, and Paiboon Jungsuwadee

Department of Toxicology and Cancer Biology (J.D., D.C., L.C., W.Z., D.St.C., M.V.), Division of Cardiovascular Medicine (M.S., A.J.M.), Markey Cancer Center (C.W.) University of Kentucky, Lexington, Kentucky; Department of Pathology and Laboratory Medicine, William S. Middleton Memorial Veterans Administration Hospital and University of Wisconsin Medical School, Madison Wisconsin (L.C.); and School of Pharmacy, Fairleigh Dickinson University, Florham Park, New Jersey (P.J.)

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ABSTRACT

Cardiotoxicity is a major dose-limiting adverse effect of doxorubicin (DOX), mediated in part by overproduction of reactive oxygen species and oxidative stress. Abcc1 (Mrp1) mediates the efflux of reduced and oxidized glutathione (GSH, GSSG) and is also a major transporter that effluxes the GSH conjugate of 4-hydroxy-2-nonenal (HNE; GS-HNE), a toxic product of lipid peroxidation formed during oxidative stress. To assess the role of Mrp1 in protecting the heart from DOX-induced cardiac injury, wild-type (WT) and Mrp1 null (Mrp1^{−/−}) C57BL/6 littermate mice were administered DOX (15 mg/kg) or saline (7.5 ml/kg) i.v., and heart ventricles were examined at 72 hours. Morphometric analysis by electron microscopy revealed extensive injuries in cytosol, mitochondria, and nuclei of DOX-treated mice in both genotypes. Significantly more severely injured nuclei were

observed in Mrp1^{−/−} versus WT mice ($P = 0.031$). GSH and the GSH/GSSG ratio were significantly increased in treatment-naïve Mrp1^{−/−} versus WT mice; GSH remained significantly higher in Mrp1^{−/−} versus WT mice after saline and DOX treatment, with no changes in GSSG or GSH/GSSG. GS-HNE, measured by mass spectrometry, was lower in the hearts of treatment-naïve Mrp1^{−/−} versus WT mice ($P < 0.05$). DOX treatment decreased GS-HNE in WT but not Mrp1^{−/−} mice, so that GS-HNE was modestly but significantly higher in Mrp1^{−/−} versus WT hearts after DOX. Expression of enzymes mediating GSH synthesis and antioxidant proteins did not differ between genotypes. Thus, despite elevated GSH levels in Mrp1^{−/−} hearts, DOX induced significantly more injury in the nuclei of Mrp1^{−/−} versus WT hearts.

Introduction

Doxorubicin (DOX) is a broad-spectrum and effective chemotherapeutic agent, but its use in oncologic practice is limited by dose-dependent cumulative cardiotoxicity, which results in irreversible and often fatal drug-induced congestive heart failure (Octavia et al., 2012). DOX-induced cardiotoxicity is mediated in large part by overproduction of reactive oxygen species (ROS), resulting in oxidative stress and cardiac tissue injury (Olson and Mushlin, 1990). 4-Hydroxy-2-nonenal

(HNE) is an α,β -unsaturated hydroxyalkenal and a highly electrophilic lipid peroxidation product formed during oxidative stress (Eckl et al., 1993). HNE exerts its toxicity primarily by reacting with tissue nucleophiles, including cellular proteins and glutathione (GSH) (Eckl et al., 1993). The conjugation of HNE with GSH (GS-HNE) forms a less toxic metabolite, but its intracellular accumulation can still generate toxicity resulting from end-product inhibition of relevant glutathione-S-transferases (GST), with subsequent accumulation of HNE and ensuing toxicity (Diah et al., 1999; Renes et al., 2000).

Multidrug resistance associated protein 1 (Mrp1/Abcc1), a member of subfamily C of the ATP-binding cassette (ABC) transporters, mediates ATP-dependent efflux of a wide range of chemotherapeutic drugs, such as epipodophyllotoxins and *Vinca* alkaloids, either by transporting them with reduced GSH or as GSH-glucuronide or -sulfate conjugates (Cole et al., 1992). Whereas human MRP1 mediates efflux of anthracyclines

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¹Current affiliation: College of Pharmaceutical Sciences, Southwest University, Chongqing, China.

²Current affiliation: Forensic Fluids Kalamazoo, Michigan.
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ABBREVIATIONS: ABC, ATP-binding cassette; ANOVA, analysis of variance; Cu,ZnSOD, Cu,Zn-superoxide dismutase; DOX, doxorubicin; ECSOD, extracellular superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCLc, glutamate-cysteine ligase catalytic subunit; GCLm, glutamate-cysteine ligase regulatory subunit; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GS-HNE, 4-hydroxy-2-nonenal glutathione conjugate; GST, glutathione-S-transferases; HNE, 4-hydroxy-2-nonenal; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; mAb, monoclonal antibody; MBB, monobromobimane; MnSOD, Mn-superoxide dismutase; MRM, multiple reaction monitoring; Mrp1/Abcc1, multidrug resistance-associated protein 1; pAb, polyclonal antibody; ROS, reactive oxygen species; WT, wild type.

such as DOX, murine Mrp1 does not, as a result of key differences in the amino acid sequences (Zhang et al., 2001). Mrp1 also effluxes many endogenous compounds, including GSH and GSSG (Cole et al., 1992), and tissues of Mrp1^{-/-} mice have higher GSH levels, attributed to loss of Mrp1^{-/-} mediated efflux (Lorico et al., 1997). We therefore questioned whether deletion of Mrp1 and the resultant increase in intracellular GSH might protect the heart against DOX-induced oxidative stress.

Wojnowski et al., (2005) reported that patients with a single nucleotide polymorphism in *MRP1*, G671V, have an increased risk of DOX-induced cardiotoxicity. We expressed this G671V variant in HEK293 cells and demonstrated that its $V_{\max}$ for efflux of GS-HNE was decreased 85% relative to wild-type (WT) MRP1. We also showed that Mrp1 is the sole mediator of ATP-dependent transport of GS-HNE in mouse cardiac sarcolemmal vesicles (Jungsuwadee et al., 2012). Taken together, these data suggest that Mrp1 could play a role in oxidative stress-induced injury in cardiac tissue and led us to postulate that deficiency of Mrp1 enhances DOX-induced cardiotoxicity due to intracellular accumulation of GS-HNE. Here we assessed the potential cardioprotective role of Mrp1 by comparing cardiac injury induced by a single dose of DOX in Mrp1-deficient mice (Mrp1^{-/-}) to that in WT mice and quantitated retention of GS-HNE, the cellular antioxidants GSH, GSSG, and the expression of antioxidant proteins in treatment-naïve WT and Mrp1^{-/-} mice and after treatment with saline or DOX. Surprisingly, deletion of Mrp1 did not increase GS-HNE in the hearts of treatment-naïve Mrp1^{-/-} versus WT mice; further, GS-HNE was only modestly increased in Mrp1^{-/-} versus WT hearts after DOX treatment. Nevertheless, despite increased levels of GSH in the heart, DOX induced significantly more nuclear injury in Mrp1^{-/-} versus WT mice.

Materials and Methods

Chemicals

High-performance liquid chromatography (HPLC)-grade solvents (acetonitrile, methanol, water, and acetic acid) were supplied by Fisher Scientific (Fair Lawn, NJ). Doxorubicin HCl (50 mg/25 ml) was obtained from Pfizer Laboratories (Division of Pfizer Inc, New York, NY), *d*³-GS-HNE from Cayman Chemical Company (Ann Arbor, MI) and GSH and GSSG from Sigma-Aldrich (St. Louis, MO). Antibodies were obtained as follows: mouse anti-catalase monoclonal antibody (mAb; sc-271803), rabbit anti-Cu,Zn superoxide dismutase (Cu,ZnSOD) polyclonal antibody (pAb; sc-11407), and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) pAb (sc-25778) from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-glutamate-cysteine ligase catalytic subunit (GCLc) pAb (ab80841), rabbit anti-glutamate-cysteine ligase regulatory subunit (GCLm) pAb (ab8144), rabbit anti-glutathione reductase (GR) pAb (ab16801) from Abcam Inc. (Cambridge, MA), and rabbit anti-Mn superoxide dismutase (MnSOD) pAb from Upstate (Lake Placid, NY). Mouse anti-P-glycoprotein mAb (517310) was purchased from Millipore (Bedford, MA), and anti-rat Ig-horseradish peroxidase (HRP), anti-rabbit Ig-HRP, and anti-mouse Ig-HRP from Amersham Biosciences (Piscataway, NJ). Rabbit anti-extracellular superoxide dismutase (ECSOD) pAb was a generous gift from Dr. Ladislav Dory (University of North Texas, Denton, TX).

Animals

C57BL/6 mice and Mrp1-deficient C57BL/6 littermates, initially a gift from Dr. Gary Kruh, were bred inhouse and backcrossed for at

least 10 generations before use. Mrp1^{-/-} mice will be available at The Jackson Laboratory Repository, JAX Stock No. 028129. Mice were maintained in the Division of Laboratory Animal Resources facility and provided food and water ad libitum. All experiments complied with the requirements of the Institutional Animal Care and Use Committee of the University of Kentucky (Lexington, KY). Mrp1^{-/-} male mice (10–12 weeks old) and their WT age-matched littermates (*n* = 6–8) were treated with normal saline (7.5 ml/kg) or DOX (15 mg/kg) i.v., and heart tissues were removed 72 hours after treatment for analyses.

Morphometric Quantification by Electron Microscopy

Heart tissue from the left ventricle was fixed, embedded, and processed for electron microscopy as described (Chaiswing et al., 2004). Embedded blocks from each mouse were sectioned and transferred to copper grids. Only longitudinal sections of cardiac muscle were used for examination. Grids were observed in an electron microscope (Hitachi H-600) operated at 75 kV. Random sampling was achieved by scanning the grid at low magnification so that cell injury was not apparent, yet gross sample artifacts (folds in tissues, dust particles, etc.) could be avoided. Grids were systematically scanned from top to bottom and from left to right so that photographs of entire cardiomyocytes were taken at 8000× magnification every 10–15 grid fields. Sixty individual cardiomyocytes were photographed for each treatment group. All quantitative ultrastructural data (mitochondrial, cytoplasmic, and nuclear damage) were analyzed from the same cardiomyocyte. Mitochondrial damage included mitochondrial swelling, mitochondria with the presence of myelin figures, mitochondria with loss of cristae, degeneration of mitochondria with disorganized cristae, lysosomal degradation of mitochondria, vacuolization in mitochondria, and mitochondrial membrane disruption. Mitochondria with any or several of the preceding ultrastructural criteria were used for determination of mitochondrial damage. The data for mitochondrial damage are presented as the average of the area exhibiting mitochondrial damage divided by the total area of mitochondria analyzed. Cytoplasmic damage included myofibrillar disorganization, intracytoplasmic vacuolization, intracellular edema, the presence of myelin figures, and disruption of cell membranes. Cytoplasm with any or several of these ultrastructural criteria was used for identification of cytoplasmic damage. Cytoplasmic damage data are presented as the average of the area exhibiting cytoplasmic damage divided by the total cytoplasmic area. The following ultrastructural features were used to identify injury and early apoptotic changes in nuclei: abnormal shape and size of nucleus, increased numbers and/or enlargement of nucleoli, nucleolar segregation (separation of granular and fibrillar components), nucleolar fragmentation, euchromatin condensation, and increased width of heterochromatin beneath the nuclear membrane. The total area and damaged areas of each subcellular compartment were measured in μm^2 using image J analysis software (National Institutes of Health), whereas the percentage of cells with injury and/or early apoptotic changes in nuclei was determined in digital images following electron microscopy.

Measurement of GS-HNE by Liquid Chromatography-Tandem Mass Spectrometry

Tissue homogenates were prepared by homogenizing approximately 50 mg of heart ventricle in 400 μl of distilled water in a borosilicate tube at 4°C, followed by the addition of 50 μl of 1 μM *d*³-GS-HNE. The chemical purity of *d*³-GS-HNE was confirmed by liquid chromatography-mass spectrometry (LC-MS). Proteins were precipitated by addition of 1600 μl of ice-cold acetonitrile, followed by mixing for 5 minutes and centrifugation at 4000 rpm for 10 minutes. The supernatant was transferred to a 4-ml glass vial and dried under nitrogen at room temperature. The dried samples were reconstituted in 100 μl of acetonitrile: H₂O (50:50), mixed, allowed to stand at room temperature for 10–15 minutes, and centrifuged at 4000 rpm for

5 minutes. The supernatants were then transferred to autosampler vials.

Analysis of GS-HNE was carried out using a Shimadzu HPLC coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. The samples were separated on a Machery-Nagel Nucleodur C8 Gravity column (2.0 mm $\times$ 125 mm, 5 μ m) by gradient elution with water containing 0.05% formic acid (solvent A) and acetonitrile/water (95:5) containing 0.05% formic acid (solvent B) according to the following program: 0% solvent B for the first 1 minute, then linear to 100% solvent B within the next 3 minutes, and maintained at 100% B for the last 2 minutes. The column was equilibrated back to initial conditions over 3 minutes. The flow rate was 0.5 ml/min with a column temperature of 30°C. The sample injection volume was 10 μ l. The MS was operated in the positive electrospray ionization mode with optimal ion source settings with a declustering potential of 71 V, entrance potential of 10 V, collision energy of 21 V, collision cell exit potential of 8 V, curtain gas of 20 psi, ion spray voltage of 5500 V, ion source gas1/gas2 of 40 psi, and temperature of 550°C. MRM transitions monitored were as follows: 464.2/308 and 464.2/446; d^3 -GS-HNE was used as an internal standard for quantitation of GS-HNE with MRM ion transitions of 467.2/308.1 and 467.2/449.

Preparation of Heart Ventricle for Biochemical Assays

The heart was extracted from the chest cavity; atrium, attached fat tissue and vessels were removed; and the ventricle was minced and rinsed with ice-cold buffer A consisting of 0.225 mol/liter mannitol, 0.075 mol/liter sucrose, 1 mmol/liter EGTA, and protease inhibitors (1 mmol/liter phenylmethyl sulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin). The ventricle was homogenized in 10 volumes of buffer A using a VWR Powermax mixer in borosilicate tubes. The homogenate was divided into aliquots, snap-frozen in liquid nitrogen, and stored at -80°C . The protein concentration of ventricle homogenates was determined using the bicinchoninic acid protein assay (Pierce, Thermo Scientific, Rockford, IL) and bovine serum albumin as standard.

Immunoblot Assays. For detection of catalase, GCLc, GCLm, GR, Cu, Zn-SOD, MnSOD, and EC-SOD, ventricle homogenates were fractionated on 4%–12% SDS-PAGE gels. For detection of expression of Abcb1, sarcolemmal membranes were prepared from the ventricle exactly as described (Jungsuwadee et al., 2006), and proteins were fractionated on a 6% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes, and the blots blocked in TBS/5% nonfat milk/0.1% Tween 20 (pH 7.5) followed by overnight incubation at 4°C with different primary antibodies: mouse anti-catalase monoclonal antibody (1:3,000), rabbit anti-GCLc polyclonal antibody (1:1000), rabbit anti-GCLm pAb (1:1000), rabbit anti-GR pAb (1:1,000), rabbit anti-Cu, ZnSOD pAb (1:5000), rabbit anti-MnSOD pAb (1:5,000), anti-ECSOD pAb (1:20,000), and anti-Pgp mAb (1:2000). GAPDH (1:5000) was used as the protein-loading control. The membranes were probed with appropriate secondary antibodies conjugated to horseradish peroxidase at room temperature for 1 hour and protein visualized by Enhanced Chemiluminescence Plus (RPN2236, GE Healthcare, Little Chalfont, UK).

Assay of GSH and GSSG by HPLC. The HPLC assay for GSH and GSSG is based on that developed by Senft et al. (2000), and is used widely (Yamazaki et al., 2008, 2012; Yamazaki and Villarreal, 2011; Kennedy et al., 2013; Barajas-Espinosa et al., 2014). Mouse heart homogenate was diluted with 0.5 volume of buffer A containing 15% metaphosphoric acid and mixed thoroughly, followed by centrifugation at 12,000 rpm for 15 minutes at 4°C . For GSH measurement, the supernatant was added to redox quenching buffer (20 mM HCl, 5 mM diethylenetriaminepenta-acetic acid, and 10 mM ascorbic acid) and then mixed with monobromobimane (MBB; prepared in HPLC-grade acetonitrile) derivatization buffer to yield final concentrations of 30 mM NaOH, 250 mM diethylenetriaminepenta acetic acid, and 2.5 mM

MBB (Senft et al., 2000). Derivatization of GSH with MBB was carried out at 45°C for 15 minutes, and the reaction was stopped by the addition of 0.6 N HCl. GSSG was derivatized by the same method after pretreatment of the heart homogenate with 0.5 mM N-ethylmaleimide to conjugate free GSH and reduction of GSSG to GSH by the addition of 5 mM dithiothreitol. The MBB-derivatized samples were centrifuged and the supernatants assayed for thiol-bimane fluorescence by HPLC using a linear gradient from 0–100% solvent B (50% methanol and 0.25% acetic acid in water) in solvent A (10% methanol and 0.25% acetic acid in water) within 28 minutes at a flow rate of 0.8 ml/min with fluorescence detection at Ex₃₇₀/Em₄₈₅, detected with the Waters 2475 Multi λ fluorescence detector as described (Senft et al., 2000). Fluorescence intensities versus time of elution were quantified using Waters Breeze chromatography software v.3.2 (Waters Corporation, Milford, CT), and peak areas were integrated and converted to nanomolar equivalents according to the GSH and GSSG standard curves. We used authentic GSH (G-4251, Sigma-Aldrich) and GSSG (150568, Sigma-Aldrich) to develop the standard curves for these assays, and the quantitated experimental samples had the same retention times for GSH and GSSG as the authentic standards, thus ensuring that any contaminating side products were not included in the assay. GSH/GSSG ratios in treatment-naïve and saline treated WT hearts were comparable to those obtained by others in normal mouse heart (GSH/GSSG = 26, Watanabe et al., 2013; GSH/GSSG = 18, Han et al., 2009), thus supporting the validity of the assay.

Statistical Analysis

All data are expressed as the mean $\pm$ S.E. for $n = 3$ –11 mice per group, as detailed in the figure legends. In studies comparing two groups, statistical analysis was performed with the Welch's t test. In studies comparing more than two groups, we first used Bartlett's test to test homogeneity of variance across all groups. If the Bartlett's test result was not significant, further statistical analysis was performed using a one-way analysis of variance (ANOVA) with post hoc analysis by the Newman-Keuls method. If the Bartlett's test result was significant, further statistical analysis was performed using Welch's t tests for pairwise comparisons between groups of interest with the Bonferroni correction for multiple comparison adjustment.

Results

DOX-Induced Ultrastructural Damage in Mouse Heart. We used a morphometric analysis of electron micrographs of the heart after saline versus DOX treatment to identify potential differences in WT versus Mrp1 $^{-/-}$ mice. This ultrastructural pathologic examination demonstrated that DOX treatment caused significant damage to cardiomyocytes. Cytoplasm, mitochondrial, and nuclear injury predominated in both genotypes after DOX treatment. Loss of mitochondrial cristae and swollen mitochondria were exceedingly rare in saline-treated mice, whereas DOX-treated mice showed dramatic subcellular changes, including mitochondrial vacuolization, the presence of myelin figures, cytoplasmic vacuolization, myofibril disarray, and mitochondrial degeneration (Fig. 1, A–D). DOX-induced mitochondrial damage was greater than cytoplasmic damage in both genotypes. Total damaged areas of cardiac tissue were comparable between WT and Mrp1 $^{-/-}$ mice after DOX treatment and were significantly different from that of saline controls (Fig. 1, E–G). In contrast, Mrp1 $^{-/-}$ mice treated with DOX demonstrated significantly more severe nuclear injuries relative to their WT counterparts (Fig. 2E), and nuclear injuries were also increased compared with saline controls. DOX caused an abnormal size and shape of nucleoli and formation of condensed chromatin, the latter

Fig. 1. Representative electron micrographs (8000×) demonstrating ultrastructural lesions identified in mouse hearts. Shown are results in mice treated with DOX (A) in WT + saline, (B) WT + DOX, (C) Mrp1^{-/-} + saline, (D) Mrp1^{-/-} + DOX; and the quantitative analysis of damaged areas: (E) cytoplasmic damage, (F) mitochondrial damage, (G) total damage. Significant pathologic changes identified include mitochondria with myelin figures (arrowheads), intracellular vacuolization (*), mitochondrial degeneration (arrows), and myofibril disarray (D). M, mitochondria; Myo, myofibril. Each bar represents the mean ± S.E. (n = 6). *P < 0.05 DOX versus saline of the same genotype; #P < 0.05 versus respective WT mice by Welch's t test.

indicative of early apoptotic changes. The presence of nucleolar fragmentation and segregation of fibrillar and granular components (Fig. 2, A–D) provided further evidence of severely damaged nuclei.

GSH and GSSG Concentration in Mouse Heart. GSH is the most abundant endogenous antioxidant that plays an important role in protecting cells from oxidative damage. A decrease in the GSH/GSSG ratio provides a measure of the degree of oxidative stress in a biologic system. Here we examined hearts from treatment-naïve mice and after treatment with saline or DOX and quantitated GSH and GSSG levels to assess oxidative stress. Basal levels of GSH in treatment-naïve mice were significantly higher in heart of Mrp1^{-/-} versus WT mice (27.8 μmol/g protein vs. 17.7 μmol/g protein, $P = 0.0002$), whereas there was no difference in the GSSG levels, such that the baseline GSH/GSSG ratio in Mrp1^{-/-} mice was significantly ($P = 0.0015$) higher than that in WT mice (Fig. 3A). GSH concentrations in heart of Mrp1^{-/-} mice remained significantly higher than WT mice after saline and DOX treatment (Bonferroni adjusted $P = 0.0204$ and 0.0028 , respectively) (Fig. 3B). GSSG levels were comparable 72 hours after DOX treatment in WT and Mrp1^{-/-} hearts (Fig. 3B), as were the GSH/GSSG ratios.

GS-HNE in Mouse Heart. Oxidative stress mediated by DOX causes lipid peroxidation with HNE as one of the major toxic lipid metabolites. The highly electrophilic HNE reacts rapidly with GSH to form GS-HNE. Despite its decreased reactivity, GS-HNE remains toxic and requires elimination by an efflux transporter. We measured GS-HNE (nmol/g heart tissue) in the heart of treatment-naïve mice using LC-MS/MS and found that whereas GS-HNE was significantly higher in treatment-naïve WT versus Mrp1^{-/-} mice ($P < 0.05$; Fig. 4A), GS-HNE levels were comparable in saline-treated WT and Mrp1^{-/-} mice. GS-HNE was unexpectedly decreased in WT mice after DOX ($P = 0.0003$), whereas GS-HNE in Mrp1^{-/-} mice remained unchanged after DOX, so that GS-HNE

detected in the heart of Mrp1^{-/-} mice was significantly higher than that in WT mice ($P < 0.0001$; Fig. 4B).

Expression of Antioxidant Proteins. Under oxidative stress conditions, cells typically respond by increasing biosynthesis and maintenance of GSH, as shown in Fig. 3, after DOX treatment in Mrp1^{-/-} heart. We analyzed protein expression of GCLc, GCLm, and GR in hearts isolated from WT and Mrp1^{-/-} mice 72 hours after treatment with saline or DOX. DOX significantly increased the expression of GCLm in both WT and Mrp1^{-/-} mice compared with saline controls ($P < 0.05$). No differences were seen in either GCLc or GR protein expression in WT or Mrp1^{-/-} mice treated with saline or DOX (Fig. 5).

The expression of several antioxidant enzyme genes is altered after DOX treatment (Li et al., 2000). Therefore, we examined protein expression of catalase, Cu, ZnSOD, MnSOD, and ECSOD in WT and Mrp1^{-/-} mice after saline or DOX treatment. Compared with saline-treated mice, catalase expression increased in response to DOX treatment in both WT and Mrp1^{-/-} mice ($P < 0.05$, Fig. 5); however, no statistically significant difference was observed between genotypes, nor was any change in expression of any of the three isoforms of SOD enzymes after DOX treatment, regardless of genotypes (Fig. 5).

Discussion

The present study clearly demonstrates that DOX-induced acute cardiac toxicity was significantly exacerbated in Mrp1^{-/-} mice. Whereas morphometric analysis of electron micrographs of the heart showed significant damage to the mitochondria and the cytoplasm after DOX treatment, consistent with previous findings (Yen et al., 1996), this injury was not different in the two genotypes. In contrast, the nucleus showed significantly more damage after DOX treatment of Mrp1^{-/-} mice, with fragmentation of the nucleolus, segregation of granular and

Fig. 2. Representative nuclear features in the heart of mice treated with DOX. (A) WT + DOX; uninjured nucleus. Areas containing granular (G) and fibrillar (F) components are distributed randomly throughout the nucleolus. (B) WT + DOX, slightly injured nucleus. Clear segregation of the granular and fibrillar components (arrow) and fragmentation of the nucleolus (arrowhead) have taken place. (C) Mrp1^{-/-} +DOX; severely injured nucleus. Enlarged nucleolus with segregation of the granular components (arrow) restricted to the central portion of the nucleolus and the fibrillar component (arrowhead) with relatively high electron density. (D) Mrp1^{-/-} +DOX; early apoptotic nucleus (arrow). Nucleolus is condensed (C) with adjacent densely compacted chromatin (CC). (E) Quantitative analysis of damaged nuclei. Each bar represents mean $\pm$ S.E. $n = 6$. * $P < 0.05$ DOX versus saline of the same genotype; # $P < 0.05$ Mrp1^{-/-} versus respective WT mice by Newman-Keuls multiple comparison test after one-way ANOVA.

fibrillar components, and condensation of nucleoli with compacted chromatin. The nucleolus is the primary site of transcription, assembly, and processing of cellular RNA (Antoniali et al., 2014), with a tripartite organization that reflects the different steps of ribosomal biogenesis. RNA polymerase I transcription starts in the fibrillar center, with the dense fibrillary component the site of initial stages of pre-rRNA processing and a granular component involved in the late processing steps. DOX has been shown to inhibit ribosomal RNA synthesis at the initial step of rRNA transcription, which in turn leads to nucleolar disintegration (Burger et al., 2010). The present findings of nucleolar disintegration and nuclear injury are consistent with DOX actions and indicate a significantly greater susceptibility to injury in Mrp1^{-/-} mice. Although we also observed a greater increase in cytoplasmic injury after saline treatment in Mrp1^{-/-} versus WT mice (Fig. 1), such an increase was not observed in an earlier study that demonstrated a significant increase in nuclear injury in DOX-treated Mrp1^{-/-} versus WT mice (Coy, 2012), consistent with the present studies.

Fig. 3. (A) GSH, GSSG, and the GSH/GSSG ratio in treatment-naïve mouse heart ($n = 8$) and (B) in hearts of saline- or DOX-treated mice ($n = 6$). Data represent mean $\pm$ S.E. * $P < 0.05$ by Welch's t test.

Whereas the mechanism by which DOX induces cardiac injury is multifactorial, it is well accepted that DOX-induced oxidative stress is a significant contributor to cardiotoxicity. DOX is an anthracycline that readily binds to cardiolipin and is thus concentrated in mitochondria, where its quinone moiety undergoes a one-electron reduction to form the semi-quinone, which in turn reduces molecular oxygen, generating the superoxide radical (Olson and Mushlin, 1990). Because the quinone moiety is capable of undergoing redox cycling, one molecule of DOX can generate many molecules of superoxide ($O_2^{\bullet-}$), which can be converted to hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and peroxynitrite. The ensuing oxidative stress is countered by a number of antioxidants in the heart, with GSH serving as a major antioxidant. GSH in Mrp1^{-/-} mice was significantly elevated, consistent with previous reports demonstrating that loss of Mrp1 and Mrp1-mediated efflux increases tissue GSH (Lorico et al., 1997). GSH is the most abundant endogenous thiol-containing antioxidant molecule and can directly scavenge strong oxidants such as $O_2^{\bullet-}$ and peroxynitrite in a nonenzymatic manner. GSH is also a cofactor for GSTs that catalyze conjugation with electrophilic products of lipid peroxidation such as HNE and serves as a cofactor for glutathione peroxidase in the reduction of H_2O_2 (Backos et al., 2012). Thus, maintenance of GSH and a stable GSH/GSSG ratio are beneficial in maintaining low cellular levels of ROS, decreased lipid peroxidation, and production of HNE. The decreased GS-HNE detected in treatment-naïve Mrp1^{-/-} mice may thus reflect the antioxidant protection provided by increased GSH,

Fig. 4. GS-HNE concentration in the hearts of (A) treatment-naïve and (B) saline- or DOX-treated mice. Data represent mean $\pm$ S.E. Left panel; $n = 8$ per group; $*P < 0.05$ by Newman-Keuls multiple comparison test after one-way ANOVA. Right panel; $n = 11$ WT; $n = 8$ Mrp1^{-/-}; $*P < 0.05$ by Welch's t test.

resulting in lower basal levels of lipid peroxidation. Even after DOX treatment, GSH levels remained elevated at 72 hours in Mrp1^{-/-} mice relative to WT mice. Ongoing studies in cultured cardiomyocytes (data not shown) from WT and Mrp1^{-/-} mice show a rapid depletion of GSH within 15 minutes after the addition of DOX, with a corresponding increased GSSG that occurs to the same extent in both genotypes. However, whereas GSH and GSSG return to that in saline controls in WT cells within 2–4 hours, in Mrp1^{-/-} cells, GSSG remains elevated from 15 minutes to 24 hours, and GSH rapidly returns to control levels at 1 hour, continues to increase, and is significantly increased and stable from 12 to 24 hours. The GSH/GSSG ratio is thus significantly decreased at 15 minutes, recovers, and does not differ between genotypes. These data are consistent with the findings in heart

tissue (Fig. 3), where GSH is significantly greater in DOX-treated Mrp1^{-/-} mice versus WT mice at 72 hours, and the GSH/GSSG ratio does not differ between treatment or genotype. The rapid recovery of GSH implies a more rapid rate of synthesis. Glutamate-cysteine ligase (GCL) catalyzes the rate-limiting step in de novo GSH synthesis and is composed of two subunits, a catalytic subunit, GCLc, and a modifier subunit, GCLm, that interacts with GCLc to greatly enhance its catalytic efficiency (Franklin et al., 2009). Mice deficient in GCLm exhibit decreased GSH levels and are more susceptible to oxidative stress (Cole et al., 2011). DOX treatment significantly increased expression of GCLm to a similar extent in WT and Mrp1^{-/-} mice. Increased GSH synthesis, coupled with decreased efflux, presumably accounts for the greater GSH levels after DOX treatment in Mrp1^{-/-} mice and is undoubtedly part of the adaptive response to loss of expression of Mrp1.

We also examined the effects of chronic DOX treatment in WT and Mrp1^{-/-} mice on GSH homeostasis (Zhang et al., 2015). Mice were treated with saline or 2 mg/kg DOX twice a week for 5 weeks, and GSH and GSSG were determined in the hearts 48 hours and 2 weeks after the last treatment. In this chronic treatment model, GSH and the GSH/GSSG ratio were decreased at 48 hours but had recovered by 2 weeks after treatment (Zhang et al., 2015). These data are consistent with a decrease in GSH and GSH/GSSG upon oxidative stress that in turn triggers activation of the antioxidant stress response system to increase GSH synthesis and restore a normal GSH/GSSG ratio. Taken together, these data imply that restoration of redox homeostasis has occurred by 72 hours after a single DOX dose and had not

Fig. 5. Western blot and quantitative analysis of (A) catalase, Cu, ZnSOD, MnSOD, GCLc, GCLm, GR protein and (B) ECSOD expression detected in hearts 72 hours after treatment with saline or DOX. Each bar presents the mean $\pm$ S.E. (A, $n = 3$, $*P < 0.05$ by Newman-Keuls multiple comparison test after one-way ANOVA, B, $n = 6$).

yet occurred at 48 hours but was apparent 2 weeks after chronic DOX treatment.

Among the many products of lipid peroxidation, including isoprostanes (Milne et al., 2011), HNE has been most extensively studied because of its abundance and high reactivity with cellular nucleophiles, including DNA, proteins and lipids (Eckl et al., 1993). HNE peaks 2 hours after DOX administration to rats, then declines to baseline levels within 8–24 hours (Luo et al., 1997). In mice, HNE increases as early as 3 hours, peaks at 6 hours, and subsequently declines at 24 hours (Chaiswing et al., 2004). Although we anticipated increased HNE adduction of proteins in Mrp1^{-/-} mice after DOX, we did not detect any differences in HNE-adducted proteins in heart homogenates between treatment or genotypes 72 hours after DOX treatment (data not shown); however, we detected significantly increased isoprostanes in hearts 72 hours after DOX, thus confirming DOX-induced oxidative stress and lipid peroxidation (Supplemental Fig. 1).

After perfusion of isolated rat hearts with HNE, Ishikawa et al. (1986) demonstrated a depletion of intracellular GSH accompanied by an increase in GS-HNE efflux into the perfusate by a saturable process, indicating formation of a GSH conjugate subsequently effluxed by a transporter. In the mouse heart, Mrp1 is the primary efflux transporter of GS-HNE, as shown using sarcolemmal vesicles (Jungsuwadee et al., 2012). Although we anticipated an increased retention of GS-HNE in heart of Mrp1^{-/-} mice relative to WT mice after DOX treatment, the basal level of GS-HNE in treatment-naïve Mrp1^{-/-} mice was significantly lower than that in their WT counterparts, as discussed herein. In further unexpected results, GS-HNE was decreased in heart after DOX treatment of WT mice. We demonstrated that DOX treatment increases expression of Mrp1 protein in heart that is maximal at 6 hours (Jungsuwadee et al., 2006); the decreased GS-HNE detected in DOX-treated WT mice may therefore reflect the induction of Mrp1 expression by DOX and its increased efflux. GS-HNE levels were not different in saline- versus DOX-treated hearts from Mrp1^{-/-} mice, so that GS-HNE in DOX treated Mrp1^{-/-} mice was modestly, but significantly, greater than that in DOX-treated WT mice. Again, these data suggest that complete loss of Mrp1-mediated GS-HNE efflux contributes to its increased retention in Mrp1^{-/-} mice.

In contrast, after chronic DOX treatment, GS-HNE was somewhat elevated 48 hours after DOX and significantly increased 2 weeks after the last dose of DOX in both WT and Mrp1^{-/-} mice (Zhang et al., 2015). These data imply that the early increase in Mrp1 expression is not sufficiently long-lasting to sustain low GS-HNE levels in WT mice after 5 weeks of DOX treatment. Indeed, immunoblots of heart from saline- and DOX-treated mice at 48 hours and 2 weeks after chronic DOX treatment showed no differences in expression of Mrp1 (Zhang et al., 2015).

In addition to conjugation with GSH, HNE can be detoxified by oxidation to 4-hydroxy-2-nonenoic acid and reduced to 1,4-dihydroxy-2-nonene. GS-HNE can be similarly oxidized and reduced, and it can also be metabolized to mercapturic acid conjugates (Alary et al., 2003; Volkel et al., 2005). Whether these HNE and GS-HNE metabolites are substrates for Mrp1 and how their formation is affected by loss of Mrp1 expression are not known.

Although DOX is not a substrate for murine Mrp1, we investigated whether other ABC efflux transporters might be

upregulated in Mrp1^{-/-} mice. Expression of Abcb1 in sarcolemmal membranes from mouse heart was not different between treatments or genotypes (Supplemental Fig. 2). We also quantitated DOX in heart of WT and Mrp1^{-/-} mice at 6, 12, and 24 hours after DOX administration and found no differences in DOX retention, consistent with the lack of changes in Abcb1 expression in these mice (Supplemental Fig. 3). Finally, we examined expression of Abcb1 and Abcc4/Mrp4 protein 2 weeks after chronic DOX treatment and found no changes in their expression between treatments or genotype (Zhang et al., 2015).

In summary, the important and novel finding provided by these studies is demonstration that DOX causes significantly greater nuclear injury in the heart of Mrp1^{-/-} versus WT mice, despite essentially identical concentrations of DOX and significantly higher concentrations of GSH compared with WT mice. We postulated that retention of GS-HNE, a product of DOX-induced lipid peroxidation, would lead to accumulation of HNE and significantly increase damage to cellular proteins; however, we found only a modest, albeit significant, increase in GS-HNE in DOX-treated Mrp1^{-/-} versus WT mice and did not detect a significant increase in protein HNE adducts, thus not supporting this hypothesis. Deletion of Mrp1 clearly perturbed the regulation of GSH as a result of retention of both GSH and GSSG, and this dysregulation likely contributes to the greater DOX cardiotoxicity in Mrp1^{-/-} mice. These data also suggest that Mrp1 has additional functions other than efflux of these known substrates, and whose loss contributes to toxicity. Thus, the development of MRP1 inhibitors in the treatment of cancer (Ma et al., 2014; Zhang et al., 2014) may have unanticipated toxicities, including cardiotoxicity.

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Authorship Contributions

Participated in research design: Deng, St Clair, Vore, Jungsuwadee.
Conducted experiments: Deng, Zhang, Chaiswing.
Contributed new reagents or analytic tools: Sunkara, Morris.
Performed data analysis: Deng, Zhang, Chaiswing, Vore.
Wrote or contributed to the writing of the manuscript: Deng, Coy, Zhang, Morris, Wang, Chaiswing, St. Clair, Vore, Jungsuwadee.

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Address correspondence to: Mary Vore, Department of Toxicology and Cancer Biology, University of Kentucky, 306 Health Sciences Research Building, Lexington, KY 40536. E-mail: maryv@email.uky.edu. or Dr. Paiboon Jungsuwadee, Fairleigh Dickinson University, School of Pharmacy, Florham Park, NJ 07932. E-mail: paiboon@fd.edu.