Metallothionein alleviates glutathione depletion-induced oxidative cardiomyopathy in murine hearts

Jun Ren, MD, PhD; Jamie R. Privratsky, MD; Xiaoping Yang, PhD; Feng Dong, MD, PhD; Edward C. Carlson, PhD

Objective: Antioxidant therapy has shown some promise in critical care medicine in which glutathione depletion and heart failure are often seen in critically ill patients. This study was designed to examine the impact of glutathione depletion and the free radical scavenger, metallothionein (MT), on cardiac function.

Design: Friend virus B and MT transgenic mice were given the glutathione synthase inhibitor buthionine sulfoximine (buthionine sulfoximine [BSO], 30 mmol/L) in drinking water for 2 wks.

Measurements: Echocardiographic and cardiomyocyte functions were evaluated, including myocardial geometry, fraction shortening, peak shortening, time-to-90% relengthening (TR90), maximal velocity of shortening/relengthening (±dL/dt), intracellular Ca$_2^+$ rise, sarcoplasmic reticulum Ca$_2^+$ release, and intracellular Ca$_2^+$ decay rate. Sacro (endo)plasmic reticulum Ca$_2^+$-ATPase function was evaluated by $^{45}$Ca uptake. Highly reactive oxygen species, caspase-3, and aconitase activity were detected by fluorescent probe and colorimetric assays.

Main Result: BSO elicited lipid peroxidation, protein carbonyl formation, mitochondrial damage, and apoptosis. BSO also reduced wall thickness, enhanced end systolic diameter, depressed fraction shortening, peak shortening, ±dL/dt, sarcoplasmic reticulum Ca$_2^+$ release, $^{45}$Ca uptake, and intracellular Ca$_2^+$ decay, leading to prolonged TR$_{90}$. BSO-induced mitochondrial loss and myofilament aberration. MT transgene itself had little effect on myocardial mechanics and ultrastructure. However, it alleviated BSO-induced myocardial functional, morphologic, and carbonyl changes. Western blot analysis showed reduced expression of sacro (endo)plasmic reticulum Ca$_2^+$-ATPase2a, Bcl-2 and phosphorylated GSK-3$.\beta$, enhanced calreticulin, Bax, p53, myosin heavy chain-β isozyme switch, and IκB phosphorylation in FVB-BSO mice, all of which with the exception of p53 were nullified by MT.

Conclusion: Our findings suggest a pathologic role of glutathione depletion in cardiac dysfunction and the therapeutic potential of antioxidants. (Crit Care Med 2008; 36:2106–2116)

Key Words: glutathione; oxidative stress; myocardial mechanics; metallothionein; Sarco (endo)plasmic reticulum Ca$_2^+$-ATPase

Oxidant balance is essential to the maintenance of physiologic cardiac function (1, 2). The antioxidant reserve often becomes inadequate in critically ill patients, leading to oxidative stress and myocardial dysfunction (1, 3–5). Several thiol-rich enzymatic or nonenzymatic components, such as glutathione (GSH) and metallothionein (MT), have been shown to preserve cardiac function by scavenging reactive oxygen species (ROS) under ischemic and diabetic injury (6–9), which is consistent with the beneficial response of antioxidant therapy in critical care medicine (3). GSH defends against oxidative stress through the glutathione redox cycle by accepting peroxides and peroxide-derived ROS or serving as a cofactor for antioxidant enzymes (10, 11). Depletion or loss of GSH has been commonly found in critically ill patients (12), contributing to oxidative injury (8, 13). Nonetheless, the role of GSH loss in heart failure in critically ill patients is still unknown. Given the cardiac benefit of MT, a potent thio-rich scavenger of ROS especially hydroxyl radicals (6–9), this study was designed to examine the effect of GSH depletion on myocardial function and ultrastructure in mice with cardiac-specific overexpression of MT. Buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase and the de novo GSH synthesis (14), was used to deplete GSH to elicit an irreversible oxidative damage (13). Echocardiographic, cardiomyocyte contractile, ultrastructural, and intracellular Ca$_2^+$ properties were evaluated. Doxorubicin, a potent antineoplastic agent prescribed for cancer treatment, was used as a positive control for oxidative cardiomyopathy (15). MT was reported to reverse doxorubicin-induced cardiac toxicity, including functional and morphologic integrity (6, 7), although little is known about cardiomyocyte contractile and intracellular Ca$_2^+$ properties. Oxidative stress, protein damage, mitochondrial function, and apoptosis were examined using reduced-to-oxidized GSH ratio, carbonyl formation, mitochondrial aconitase, and caspase-3 activity, respectively. To explore the mechanism of action involved in intracellular Ca$_2^+$ homeostasis and apoptosis in response to BSO and MT exposure, expression of the Ca$_2^+$ regulating proteins sarco (endo)plasmic reticulum Ca$_2^+$-ATPase (SERCA), phospholamban, calreticulin, myosin heavy chain (MHC), Kv1.2 channel, anti-apoptotic (apoptosis repressor
with caspase recruitment domain [ARC] and Bcl-2), and pro-apoptotic (p53, Bax, NFκB, and glycogen synthase kinase-3β [GSK-3β]) proteins also was monitored.

**MATERIALS AND METHODS**

**Experimental Animals, Treatment with BSO, and Doxorubicin**

The experimental protocols were approved by our institutional Animal Use and Care Committee. Five-month-old, male transgenic mice with a ten-fold MT protein overexpression driven by the cardiac-specific mouse α-MHC promoter (6) and wild-type Friend virus B (FVB) mice were maintained on a 12/12-light/dark cycle with lab chow and water ad libitum. A cohort of FVB and MT mice was given BSO (30 mmol/L) in drinking water for 2 wks (13). Another cohort of FVB and MT mice was injected with doxorubicin hydrochloride (20 mg/kg body weight, intraperitoneally) 5 days before experimentation (6). Systolic blood pressure was measured with a semiautomated, amplified tail cuff device.

**Echocardiographic Assessment.** Animals were anesthetized by using 2.5% avertin (10 μL/g body weight). Two-dimensional (2-D) guided M-mode echocardiography was performed using a Phillips echocardiography system (Sonos 5500) equipped with a 15–6 MHz linear transducer. The heart was first imaged in the 2-D mode in parasternal short-axis views, which were used to position the M-mode cursor perpendicular to ventricular septum and left ventricular (LV) posterior wall and to subsequently obtain M-mode images. Anterior and posterior wall thickness and diastolic and systolic LV dimensions were recorded from M-mode images using averaged measurements from 3 cardiac cycles with the leading edge-to-leading edge convention adopted by the American Society of Echocardiography (16). Fractional shortening was calculated from end-diastolic diameter (EDD) and end-systolic diameter (ESD). Heart rates were averaged over 10 cardiac cycles.

**Cardiomyocyte Isolation.** After ketamine/xylazine sedation, hearts were removed and perfused with Krebs-Henseleit bicarbonate buffer containing (in mmol/L): 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES, and 11.1 glucose. Hearts were digested with collagenase D for 20 mins. Left ventricles were removed and minced before being filtered (17). The percentage of the rod-shaped viable cardiomyocytes was ~70%, as determined using a hemacytometer. Neither BSO nor MT overtly affected the cardiomyocyte yield.

**Cardiomyocyte Contractile Function.** Mechanical properties of myocytes were assessed using an IonOptix soft-edge system (IonOptix, Milton, MA) (17). Myocytes were placed in a chamber mounted on the stage of an Olympus IX-70 microscope and superfused (~2 mL/min at 25°C) with a KHB buffer containing 1 mmol/L CaCl2. Myocytes were field stimulated at 0.5 Hz unless otherwise stated. Cell shortening and re-lengthening were assessed, including peak shortening (PS) – peak contractility, time-to-PS (TPS) – contraction duration, time-to-90% re-lengthening (TR envelope) – relaxation duration and maximal velocities of shortening/re-lengthening (+/−dL/dt) – maximal pressure development and decline.

**Intracellular Ca2+ and SR Ca2+**. Intracellular Ca2+ was evaluated with a dual-excitation, single-emission photomultiplier system (IonOptix) in myocytes loaded with fura-2 (0.5 μmol/L) (17). Myocytes were exposed to light emitted by a 75W halogen lamp through a 360-nm or 380-nm filter while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm and qualitative changes in intracellular Ca2+ were inferred from the ratio of fura-fluorescence intensity (FF) at 360/380 nm. Fluorescence decay was measured as an indicator for intracellular Ca2+ clearing. SR Ca2+ release was assessed by rapid puffed of caffeine (10 mmol/L) in fura-2-loaded myocytes. Multiple applications of caffeine were given at 5-min intervals to ensure a steady state.

**Reduced and Oxidized Glutathione (GSH and Glutathione Disulfide [GSSG]).** Tissue samples were homogenized and centrifuged. Supernatant fractions were collected for GSH and GSSG assay. Half of each sample was used for GSH determination and the other half for GSSG assay. Samples (100 μL) were incubated at room temperature with 2 μL 4-vinyl pyridine for 1 hr to conjugate GSH for determination of GSSG. The GSSG was then subtracted from the total glutathione to determine the GSH levels (18).

**ROS Detection.** Highly reactive oxygen species (hROS) were assessed with 2-[6-(4'-hydroxy-phenoxo)-3H-xanthene-3-ony-9-yl]benzoic acid (HPF) and 2-[6-(4'-amino)phenoxo-3H-xanthene-3-on-9-yl]benzoic acid (APF) (Molecular Probes, Eugene, OR). APF is highly specific for hydroxyl radical and hypochlorite with little reactivity toward superoxide, alkyloxy radical, oxygen radical, and nitric oxide. On the other hand, HPF is useful for hydroxyl radical. Total ROS was determined by 2',7'-dichlorodihydrofluorescein (DCF). Isolated cardiomyocytes were loaded with APF, HPF, or DCF (5 μmol/L) at 37°C for 1 hr, and fluorescent intensity was measured using a fluorescent microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 515 nm (9,19).

**Protein Carbonyl.** Protein was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) and centrifuged for 1 min. The sample resuspended in 10 mmol/L 2,4-dinitrophenylhydrazine solution for 15–30 mins at room temperature before 20% TCA was added and samples were centrifuged for 3 mins. The precipitate was resuspended in 6 mol/L guanidine solution. The maximum absorbance (580–390 nm) was read against appropriate blanks (2 mol/L HCl) and carbonyl content was normalized to respective protein content (17).

**Lipid Peroxidation End Product Malondialdehyde (MDA).** Samples were treated with butylated hydroxytoluene and were heated to 90°C for 45 mins. The mixture was centrifuged and samples were eluted with a high performance liquid chromatography system fitted with a Shimadzu SIL-10A autoinjuctor and a Waters Symmetry column (C18, 5.0 μm particle size, 4.6 mm × 250 mm) (Waters, Milford, MA). The MDA peak of interest was eluted at 8 mins and detected by using a Waters 474 scanning fluorescence detector at 532 nm excitation and 553 nm emission (18).

**Caspase-3 Assay.** Cardiomyocytes were lysed in an ice-cold cell lysis buffer before lysis with reaction buffer and caspase-3 colorimetric substrate (Ac-DEVD-pNA) at 37°C for 1 hr, during which time the caspase in the sample was allowed to cleave the chromophore p-NA from the substrate molecule. The samples were then read with a microplate reader at 405 nm. Caspase-3 activity was expressed as picomoles of pNA released per microgram of protein per minute (20).

**Aconitate Activity.** Mitochondrial fractions from heart homogenate were resuspended in sodium citrate and aconitate activity assay was performed using an Aconitate-340 assay kit from OxisResearch (Portland, OR) to measure nicotinamide adenine dinucleotide phosphate formation, a product of the oxidation of isocitrate to α-ketoglutarate (21). Mitochondrial samples (30 μl) were incubated in a 96-well plate with 50 μL trisodium citrate (substrate), 50 μL nicotinamide adenine dinucleotide phosphate for 15 mins at 37°C. The absorbance was recorded at 340 nm every minute for 5 mins with a SpectraMax 190 Microplate Spectrophotometer.

**SERCA Activity.** Cells were sonicated and solubilized in a Tris-sucrose homogenization buffer. To determine SERCA-dependent Ca2+ uptake, paired samples were treated with and without 10 μmol/L of the SERCA inhibitor thapsigargin for 15 mins. The difference between the two readings was deemed the thapsigargin-sensitive 45Ca2+ uptake through SERCA. Uptake was initiated by the addition of an aliquot of supernatant from each sample to a solution consisting of (in mmol/L) 100 KCl, 5 NaNO3, 6 MgCl2, 0.15 ethylene glycol/1 acetate (EGTA), 0.12 CaCl2, 30 Tris-Cl pH 7.0, 10 oxalate, 2 adenosine 5'-triphosphate (ATP), and 1 μCi 45CaCl2 in water bath at 37°C. Aliquots of samples were injected onto glass filters on a suction manifold, washed, and filters were collected for scintillation count. SERCA activity was expressed as cpm/mg protein of the thapsigargin-sensitive 45Ca2+ uptake (17).
Electron Microscopy. Mouse abdomens and thoraxes were opened under anesthesia, and the right atria were incised to allow the release of blood. Perfusion fixation was immediately initiated by using a saline washout. Hearts were perfused with 20 mL of warm (37°C), pH 7.6, and PIPES-buffered formaldehyde-glutaraldehyde at ~3 mL/minute and followed by 40 mL of the same fixative but chilled to 4°C. Hearts were removed immediately and left ventricular and interventricular septal tissues were collected from a 2-mm ring sliced from the midventricular region. These were further minced to 1 mm³. Fixation continued overnight at 4°C in a 10:1 fluid/tissue ratio. This was followed by rinsing in PIPES buffer + 2% sucrase (pH 7.4) and overnight postfixation in PIPES buffered 1% OsO₄ + 2% sucrose and 1.5% K₃[Fe(CN)₆]·3H₂O at room temperature. Tissue blocks were dehydrated through graded ethanol and propylene oxide, embedded in Epon/Araldite, and cured for 48 hrs at 60°C. Thin sections (silver-gray interference color) were cut on an RMC-MTXIII ultramicrotome equipped with a Diatome diamond knife. Sections were collected on naked copper (300-mesh) grids, stained with lead citrate and uranyl acetate (4% in absolute ethanol), and imaged with a Hitachi 7500 transmission electron microscope (22).

Immunoblotting and MHC Isofrom Distribution. Membrane proteins from cardiomyocytes were extracted (17). Membrane proteins (50 μg/lane) were separated on 10–15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk with a TBS-T solution for 60 mins and then incubated with primary antibodies followed by incubation with horseradish peroxidase-coupled to an anti-mouse secondary antibody. After immunoblotting, the film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad Densitometer. For MHC gel electrophoresis, homogenized heart tissue in a sample buffer (1:30) was heated for 2 mins at 95°C and chilled on ice for 5 mins before being centrifuged. Three microelots of 1:10 diluted supernatant was loaded for electrophoresis as described (21). After running, gels were fixed for a minimum of 2 hrs in 5% glutaraldehyde before being silver-stained and scanned to determine the amount of MHC-α and MHC-β. β-actin (1:5000) was used as the loading control.

Data Analysis

Data were recorded as mean ± standard error of the mean. Statistical significance (p < .05) was estimated by two-way analysis of variance or Student’s t-test with a Dunnett’s test as post hoc analysis when required.

RESULTS

General Morphometric and Echocardiographic Characteristics of Mice After BSO Treatment

BSO and MT transgene did not elicit any notable effects on body, organ weights, or blood glucose. The systolic blood pressure was moderately elevated after BSO treatment in both FVB and MT mice. As expected, BSO significantly reduced GSH levels and the GSH/GSSG ratio without altering GSSG levels in hearts, livers, and kidneys, the effect of which was not affected by MT. Interestingly, GSH depletion enhanced protein damage evaluated by carbonyl formation, which was reversed by MT. MT transgene itself did not affect the organ redox state and protein carbonyl levels (Table 1). Whereas heart rate, EDD, LV mass, and normalized LV mass were comparable among FVB and MT mice with or without BSO treatment, BSO significantly reduced ventricular thickness and enlarged ESD, leading to a decreased fraction shortening. MT transgene abrogated BSO-induced myocardial geometric and functional changes without affecting those indices by itself (Table 2).

Table 1. General feature of Friend virus B (FVB) and metallothionein (MT) mice with or without buthionine sulfoximine (BSO) treatment for 2 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FVB (19)</th>
<th>MT (19)</th>
<th>FVB + BSO (23)</th>
<th>MT + BSO (20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.9 ± 0.9</td>
<td>29.2 ± 0.7</td>
<td>27.8 ± 0.7</td>
<td>28.7 ± 0.8</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>176 ± 9</td>
<td>175 ± 9</td>
<td>171 ± 8</td>
<td>179 ± 11</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>6.31 ± 0.25</td>
<td>6.03 ± 0.33</td>
<td>6.12 ± 0.21</td>
<td>6.32 ± 0.45</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.39 ± 0.05</td>
<td>1.50 ± 0.04</td>
<td>1.37 ± 0.05</td>
<td>1.56 ± 0.06</td>
</tr>
<tr>
<td>Kidney weight (mg)</td>
<td>419 ± 22</td>
<td>431 ± 17</td>
<td>450 ± 29</td>
<td>465 ± 20</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>99.3 ± 5.5</td>
<td>102.7 ± 8.7</td>
<td>102.3 ± 6.2</td>
<td>101.7 ± 7.5</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>103.9 ± 4.9</td>
<td>104.0 ± 5.9</td>
<td>139.6 ± 6.9*</td>
<td>143.8 ± 6.6*</td>
</tr>
<tr>
<td>Heart GSH (nmol/mg tissue)</td>
<td>114.2 ± 8.1</td>
<td>118.3 ± 9.7</td>
<td>79.0 ± 7.0*</td>
<td>84.4 ± 8.0*</td>
</tr>
<tr>
<td>Heart GSSG (nmol/mg tissue)</td>
<td>46.4 ± 4.2</td>
<td>46.9 ± 5.9</td>
<td>47.9 ± 6.8</td>
<td>45.4 ± 5.4</td>
</tr>
<tr>
<td>Liver GSH/GSSG</td>
<td>2.51 ± 0.17</td>
<td>2.63 ± 0.22</td>
<td>1.77 ± 0.20*</td>
<td>1.93 ± 0.22*</td>
</tr>
<tr>
<td>Liver GSH (nmol/mg tissue)</td>
<td>229.5 ± 42.0</td>
<td>203.9 ± 47.0</td>
<td>138.8 ± 34.7*</td>
<td>131.2 ± 37.2*</td>
</tr>
<tr>
<td>Liver GSSG (nmol/mg tissue)</td>
<td>116.8 ± 22.0</td>
<td>110.8 ± 19.8</td>
<td>108.8 ± 23.7</td>
<td>110.6 ± 34.7</td>
</tr>
<tr>
<td>Liver GSH/GSSG</td>
<td>2.00 ± 0.16</td>
<td>2.05 ± 0.29</td>
<td>1.42 ± 0.27*</td>
<td>1.49 ± 0.37*</td>
</tr>
<tr>
<td>Kidney GSH (nmol/mg tissue)</td>
<td>9.4 ± 1.1</td>
<td>8.5 ± 1.4</td>
<td>6.5 ± 0.9*</td>
<td>7.1 ± 1.1*</td>
</tr>
<tr>
<td>Kidney GSSG (nmol/mg tissue)</td>
<td>8.9 ± 1.4</td>
<td>11.1 ± 4.0</td>
<td>11.0 ± 4.0</td>
<td>10.3 ± 4.4</td>
</tr>
<tr>
<td>Kidney GSH/GSSG</td>
<td>1.13 ± 0.13</td>
<td>1.04 ± 0.34</td>
<td>0.86 ± 0.21*</td>
<td>0.72 ± 0.20*</td>
</tr>
<tr>
<td>Heart protein carbonyl (nmol/mg protein)</td>
<td>1.41 ± 0.06</td>
<td>1.37 ± 0.08</td>
<td>2.14 ± 0.08*</td>
<td>1.53 ± 0.08*</td>
</tr>
</tbody>
</table>

GSH, glutathione; GSSG, glutathione disulfide. Mean ± SEM; *p < .05 vs. FVB group; 2p < .05 vs. FVB-BSO group, number of mice per group is given in parentheses.

Table 2. Echocardiographic parameters of Friend virus B (FVB) and metallothionein (MT) mouse hearts with or without buthionine sulfoximine (BSO) treatment for 2 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FVB (9)</th>
<th>MT (9)</th>
<th>FVB + BSO (7)</th>
<th>MT + BSO (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>25.1 ± 0.9</td>
<td>26.5 ± 0.6</td>
<td>26.1 ± 1.0</td>
<td>27.6 ± 1.2</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>428 ± 21</td>
<td>447 ± 18</td>
<td>447 ± 24</td>
<td>429 ± 23</td>
</tr>
<tr>
<td>Wall thickness (mm)</td>
<td>0.84 ± 0.05</td>
<td>0.91 ± 0.07</td>
<td>0.55 ± 0.04*</td>
<td>0.84 ± 0.06</td>
</tr>
<tr>
<td>EDD (mm)</td>
<td>2.80 ± 0.11</td>
<td>2.64 ± 0.18</td>
<td>2.54 ± 0.13</td>
<td>2.85 ± 0.09</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>1.17 ± 0.08</td>
<td>1.10 ± 0.11</td>
<td>1.58 ± 0.10*</td>
<td>1.19 ± 0.09</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>73 ± 1</td>
<td>82 ± 1</td>
<td>73 ± 1</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>Normalized LV mass (mg/g)</td>
<td>2.77 ± 0.03</td>
<td>3.11 ± 0.03</td>
<td>2.72 ± 0.03</td>
<td>2.68 ± 0.02</td>
</tr>
<tr>
<td>Fraction shortening (%)</td>
<td>58.5 ± 2.0</td>
<td>58.3 ± 3.1</td>
<td>38.0 ± 1.4*</td>
<td>58.2 ± 3.1</td>
</tr>
</tbody>
</table>

EDD, end-diastolic diameter; ESD, end-systolic diameter; LV, left ventricle; Mean ± SEM; *p < .05 vs. FVB group, number of mice per group is given in parentheses.
Effect of MT and BSO on Cardiomyocyte Mechanical and Fluorescent Properties

The cell phenotype and resting length were comparable among all mouse groups (data not shown). Myocytes from the FVB-BSO group exhibited significantly depressed PS, reduced $\pm dL/dt$, and prolonged $TR_{90}$ with comparable TPS. This finding was consistent with earlier reports of impaired cardiac contractile function after broad oxidative insults (1, 9, 17, 18, 21, 23). Similar results on myocyte mechanics were observed in mice treated with doxorubicin, which was used as a positive control because of its known cardiac toxicity (6). Interestingly, overexpression of MT abrogated both BSO- and doxorubicin-induced decrease in PS and $\pm dL/dt$ as well as prolongation in $TR_{90}$ (Fig. 1). Consistently, BSO depressed

Figure 1. Effect of buthionine sulfoximine (BSO) treatment on mechanical properties in cardiomyocytes from Friend virus B (FVB) and metallothionein (MT) mice with doxorubicin (DOX) as positive control. A, Peak cell shortening (PS). B, Maximal velocity of shortening/relengthening ($\pm dL/dt$). C, Time-to-peak shortening (TPS). D, Time-to-90% relengthening ($TR_{90}$). E, PS in response to change in stimulus frequency (0.1–5.0 Hz). PS was normalized to that obtained at 0.1 Hz of the same cell. Mean ± SEM, n = 140 (A–D) and 30–34 (E) from ten mice per group. *p < .05 vs. FVB; #p < .05 vs. FVB-BSO or FVB-DOX.
electrically stimulated intracellular Ca\(^{2+}\) rise (ΔFFI), SR Ca\(^{2+}\) release, and prolonged intracellular Ca\(^{2+}\) decay without affecting baseline intracellular Ca\(^{2+}\) levels. MT nullified BSO-induced depression of ΔFFI, reduced SR Ca\(^{2+}\) release, and prolonged intracellular Ca\(^{2+}\) decay. Baseline intracellular Ca\(^{2+}\) levels were decreased in the BSO-treated MT group. The BSO-induced intracellular Ca\(^{2+}\) responses were reminiscent of those induced by doxorubicin. To delineate the possible mechanism(s) responsible for the BSO and MT-elicited changes in intracellular Ca\(^{2+}\) homeostasis, the cardiomyocyte Ca\(^{2+}\) reuptake function was tested by using \(^{45}\)Ca\(^{2+}\) uptake. The result revealed a declined cardiomyocyte \(^{45}\)Ca\(^{2+}\) uptake in BSO- and doxorubicin-treated mice, which was restored by MT. MT itself did not alter cardiomyocyte mechanics, intracellular Ca\(^{2+}\) properties, and \(^{45}\)Ca\(^{2+}\) uptake (Fig. 2), indicating little innate effect on cardiomyocyte function by MT.

Mouse hearts beat at very high frequencies (>400/min at 37°C), whereas our baseline stimulus was 0.5 Hz (30 beats/min). To investigate possible derangement of cardiac contractile function at higher frequencies, the stimulating frequency was increased stepwise from 0.1 Hz to 5.0 Hz (300 beats/min) and PS was recorded at steady-state. Cells were initially stimulated to contract at 0.5 Hz for 5 mins to ensure steady-state before commencing the frequency protocol (at

![Graphs and images showing the effect of buthionine sulfoximine (BSO) on intracellular Ca\(^{2+}\) transients, SR Ca\(^{2+}\) release, and \(^{45}\)Ca\(^{2+}\) uptake in myocytes from Friend virus B (FVB) and metallothionein (MT) mice (doxorubicin [DOX] serves as positive control). A. Representative fura-2 transients depicting the effect of BSO in FVB and MT mouse myocytes. B. Baseline fura-2 fluorescent intensity (FFI). C. Fluorescence decay rate (Tau). D. Change of FFI in response to electrical stimulus (ΔFFI). E. SR Ca\(^{2+}\) release. F. \(^{45}\)Ca\(^{2+}\) uptake. Mean ± SEM, n = 68–76 cells (7–8 for E–F). *p < .05 vs. FVB; #p < .05 vs. FVB-BSO.)

Figure 2. Effect of buthionine sulfoximine (BSO) on intracellular Ca\(^{2+}\) transients, SR Ca\(^{2+}\) release, and \(^{45}\)Ca\(^{2+}\) uptake in myocytes from Friend virus B (FVB) and metallothionein (MT) mice (doxorubicin [DOX] serves as positive control). A. Representative fura-2 transients depicting the effect of BSO in FVB and MT mouse myocytes. B. Baseline fura-2 fluorescent intensity (FFI). C. Fluorescence decay rate (Tau). D. Change of FFI in response to electrical stimulus (ΔFFI). E. SR Ca\(^{2+}\) release. F. \(^{45}\)Ca\(^{2+}\) uptake. Mean ± SEM, n = 68–76 cells (7–8 for E–F). *p < .05 vs. FVB; #p < .05 vs. FVB-BSO.
0.1–5.0 Hz). All recordings were normalized to the PS obtained at 0.1 Hz. Myocytes from the FVB–BSO group exhibited significantly exaggerated depression from baseline (0.1 Hz) at both 0.5 Hz and 1.0 Hz without significant change at 3.0 Hz and 5.0 Hz. MT transgene itself did not alter the frequency response pattern, although it completely prevented the BSO-induced depression in PS at 0.5 Hz and 1.0 Hz. Interestingly, doxorubicin failed to duplicate BSO-elicited exaggeration of PS decline in response to increased stimulus frequency (Fig. 1E).

Effect of MT and BSO on Lipid Peroxidation, ROS, Apoptosis, and Mitochondrial Function

To explore the mechanism(s) of action in MT-elicited protection against GSH depletion-induced cardiac dysfunction, lipid peroxidation, apoptosis, and mitochondrial function, all of which are likely targets of oxidative stress (17, 24–26), were examined. BSO significantly increased levels of the lipid peroxidation end product MDA in hearts, livers, and kidneys. Although MT itself did not affect organ MDA levels, it ablated BSO-induced increase in cardiac but not hepatic or renal MDA levels. Assessment of apoptosis using caspase-3 assay also suggested that MT was capable of alleviating BSO-triggered myocyte apoptosis. To explore BSO-induced myocyte injury, cardiac aconitase levels were measured. Aconitase is an iron-sulfur enzyme located in citric acid cycle and mitochondrial aconitase is readily damaged by oxidative stress via removal of an iron from [4Fe-4S] (21). Our results showed that BSO overtly reduced aconitase activity, the effect of which was rectified by MT. Finally, measurement of total and highly reactive ROS using DCF and APF/HPF depicted significantly increased fluorescent intensity from all three probes. MT effectively antagonized the BSO-elicited ROS generation with little effect itself (Fig. 3).

Electron Microscopy

Without BSO treatment, no ultrastructural differences were observed in cardiac samples between the FVB and MT groups (Fig. 4A–B). BSO treatment in FVB mice induced extensive focal damage in both left ventricular (Fig. 4C) and intraventricular septal (data not shown) sections. This was evidenced by cytoarchitectural damage, including mitochondrial swelling and significant disruption of sarcomeres and the arrangement of cardiac contractile filaments. Consistent with the mechanical observations, MT prevented BSO-induced structural damage (Fig. 4D) and myocardial tissues from BSO-treated MT mice were ultrastructurally indistinguishable from FVB or MT samples. To examine whether the MT-elicited protection on cardiac ultrastructure can be mimicked by exogenously administered antioxidants, the effect of α-tocopherol acetate (200 mg/kg body weight administered via gavage for 2 wks)

Figure 3. A, Levels of lipid peroxidation end product malondialdehyde (MDA) in hearts, livers, and kidneys from Friend virus B (FVB) and metallothionein (MT) mice treated with or without buthionine sulfoximine (BSO). B, reactive oxygen species (ROS) measured by dichlorodihydrofluorescein (DCF), 2-[6-(4′-amino)phenox-3H-xanthen-3-on-9-yl]benzoic acid (APF), and 2-[6-(4′-hydroxy)phenox-3H-xanthen-3-on-9yl]benzoic acid (HPF) fluorescence probes. C, Caspase-3 activity. D, Aconitase activity in hearts from FVB and MT mice treated with or without BSO. Mean ± standard error of the mean, n = 6–9. *p < .05 vs. FVB, #p < .05 vs. FVB-BSO group.
Figure 4. Transmission electron microscopic micrographs of left ventricular tissues from Friend virus B (FVB) and buthionine sulfoximine (BSO)-treated mice with or without metallothionein (MT) transgene or vitamin E treatment. A, FVB; B, MT; C, FVB-BSO; D, MT-BSO; E, FVB + vitamin E; F, FVB-BSO + vitamin E; G, MT-doxorubicin; and H, mitochondrial number. Mean ± SEM from 5–7 fields. Tissues in A, B, D, E, and F appear normal with myofibrils composed of regular and uninterrupted sarcomeres separated by continuous rows of normal mitochondria. BSO-treated myocardial tissues (C) displayed irregularly shaped mitochondria, myelin figures, and highly disrupted myofibrils.
on BSO-induced ultrastructural changes was evaluated (27). Similar to MT transgene, α-tocopherol acetate effectively ameliorated BSO-induced cardiac ultrastructural damage (Fig. 4F) while exhibiting little ultrastructural effect in the FVB mice (Fig. 4E), indicating a favorable response of antioxidants against GSH depletion. α-tocopherol also improved cardiomyocyte contractile function and intracellular Ca\(^{2+}\) homeostasis in BSO-treated FVB mice (data not shown), consistent with its beneficial role in cardiac oxidant injury, such as sepsis (5, 27). In addition, reminiscent of its action against BSO as reported previously (6), MT transgene also protected against doxorubicin-induced toxicity on cardiac morphology (Fig. 4G). Furthermore, BSO significantly reduced mitochondrial density in FVB hearts, the effect of which was nullified by MT (Fig. 4H).

**Immunoblotting for SERCA2a, Phospholamban, Calreticulin, MHC Isozyme, and Kv1.2**

To understand the mechanism behind changes in intracellular Ca\(^{2+}\) homeostasis in MT-elicited protection against BSO treatment, expression of several key intracellular Ca\(^{2+}\) proteins was evaluated. The immunoblotting data revealed that BSO treatment significantly reduced expression of the Ca\(^{2+}\) re-sequestering protein SERCA2a and the SERCA2a/phospholamban ratio and upregulated the endoplasmic reticulum chaperone calreticulin and MHC-β distribution without affecting levels of phospholamban and Kv1.2. Although MT itself did not alter protein expression of these Ca\(^{2+}\) regulating proteins, it nullified BSO-induced changes in SERCA2a, SERCA2a/phospholamban ratio, calreticulin, and MHC isozyme redistribution (Fig. 5).

**Immunoblotting for ARC, p53, Bax, Bcl-2, Phosphorylation of IκB, and GSK-3β**

Our data further depicted upregulated proapoptotic proteins Bax and p53, downregulated anti-apoptotic protein Bcl-2, enhanced phosphorylation of the NFKB inhibitory subunit IκB (which removes its inhibition on NFKB), and diminished GSK-3β phosphorylation in response to BSO treatment. The expression of total IκB and GSK-3β was not altered (data not shown). Alterations in these proteins are closely associated with heart failure and oxidative diseases (28, 29). Although MT itself did not affect expression of these proteins or its phosphorylation, it nullified BSO-induced alteration in Bax, Bcl-2, phosphorylation of IκB, and GSK-3β with the exception of p53. Levels of the anti-apoptotic protein ARC were not affected by BSO or MT (Fig. 6).

**DISCUSSION**

Both tissue GSH depletion and high prevalence of heart failure are commonly seen in critically ill patients (4, 12, 30), although the link between the two has never been elucidated. Our data revealed for the first time that GSH depletion leads to oxidative stress, protein carbonyl damage, lipid peroxidation, cardiac geometric and contractile defect, and ultrastructural derangement in the hearts, suggesting a potential role of GSH depletion and oxidative stress in the development of heart failure in critically ill patients. The study further indicated that SERCA dysfunction, MHC isozyme switch, enhanced pro-apoptotic, and reduced anti-apoptotic protein abundance may be responsible for the GSH depletion-induced myocardial geometric, contractile, intracellular Ca\(^{2+}\), and morphologic changes. The loss of ultrastructural integrity in conjunction with reduced mitochondrial density and aconitase activity further depicted that the myopathy may be focal to cardiomyocytes and mitochondria, likely targets for oxidative stress (9, 21, 25). The fact that the thiol antioxidant MT abrogates BSO-induced functional, ultrastructural, and mitochondrial changes supports the favorable role of antioxidant therapy in critical care medicine (3, 5).

Our results revealed that GSH depletion resulted in depressed myocardial contraction, prolonged relaxation and intracellular Ca\(^{2+}\) clearing, reduced SR Ca\(^{2+}\) release capacity, and exaggerated PS decline at higher frequencies, consistent with previous reports of compromised myocardial function under oxidant injury and critical illness, such as sepsis and shock (2, 4, 31). These cardiomyocyte contractile and intracellular Ca\(^{2+}\) defects also were reminiscent of the hallmark cardiomyocyte dysfunction in diabetic and hypertrophic cardiomyopathy, in which oxidative stress plays a key role (17, 23). The deleterious cardiac effects of GSH depletion is believed to be mediated by ROS, especially hROS independent of H\(_2\)O\(_2\), NO, and O\(_2^-\) (shown by APP/HPF). This is supported by our data that MT nullifies GSH depletion-induced ROS especially hROS accumulation, protein damage, and lipid peroxidation, which are in line with the favorable response of MT on cardiac function and morphology.

Several mechanisms may be postulated for the BSO-induced detrimental cardiac defects and MT-offered cardioprotection. First, BSO may impair cardiac function via a ROS-mediated inhibition of intracellular Ca\(^{2+}\) recruitment (32). ROS has been shown to disrupt myocardial Ca\(^{2+}\) homeostasis through inhibition of L-type Ca\(^{2+}\) currents, SR Ca\(^{2+}\) load, SERCA function, and Na\(^+\)-Ca\(^{2+}\) exchange (2, 33). Dysfunction of these Ca\(^{2+}\) regulatory elements contributes to reduced cardiac contractile function (17, 23) consistent with our findings of impaired SERCA expression/function, upregulated calreticulin expression, and compromised intracellular Ca\(^{2+}\) release and decay, as well as reduced fraction shortening, PS, ≥2.6/dt and prolonged TR90 after BSO treatment. Calreticulin, a ROS-sensitive endoplasmic reticulum chaperone, is known to sensitize SERCA to oxidative injury (34). Up-regulated calreticulin expression after BSO treatment may render the free thiol groups in calreticulin and SERCA more sensitive to oxidative protein damage (35), which is in line with the carbonyl formation data from this study. It is worth mentioning that systolic duration, resting intracellular Ca\(^{2+}\) level, phospholamban and Kv1.2 channel were not affected by BSO, indicating possible disparity in the sensitivity of cardiac contractile/Ca\(^{2+}\) regulating proteins to GSH depletion-elicited oxidative stress. Interestingly, the GSH depletion-induced depression of SERCA expression/function, up-regulation of calreticulin, myocardial mechanical, and intracellular Ca\(^{2+}\) defects were abrogated by MT in parallel with MT-elicited inhibition on ROS generation, lipid peroxida- tion, and protein carbonyl formation. These data support the notion that ROS and SERCA function may be essential to MT-elicited protection against BSO-induced cardiac mechanical and intracellular Ca\(^{2+}\) dysfunction. The culprit role of ROS in BSO-induced cardiomyocyte dysfunction was further testified by the ROS inducer doxorubicin-induced contractile and intracellular Ca\(^{2+}\) responses. Second, MHC isozyme switch from α- to β- in response to GSH depletion and MT transgene overexpression may contribute to myocardial remodeling (changes in
wall thickness and ESD) and myopathic changes in FVB and MT mice. Third, our observation of extensive ultrastructural damage, especially mitochondrial swelling and reduced mitochondrial density after chronic GSH depletion, indicates that mitochondrial integrity plays a role in BSO-induced myocardial dysfunction and MT-offered protection. The morphologic changes in mitochondria in response to BSO and MT received further support from mitochondrial aconitase activity and GSK-3β phosphorylation, which play integral roles in mitochondrial permeability transition pore (36). GSH is required to metabolize ROS generated by the mitochondria (32). Mitochondria have been considered the main targets for ROS insult and may exhibit mitochondrial permeability transition leading to mitochondrial swelling, cytochrome c release, and eventually mitochondrial loss (21, 32, 37). Earlier studies have demonstrated that MT can protect against diabetic cardiomyopathy by suppressing mitochondrial oxidative stress (24) and restoring mitochondrial GSH.

Figure 5. Western blot analysis of A, sacro (endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase2 (SERCA2): B, Phospholamban; C, SERCA2a-to-phospholamban ratio; D, calreticulin; E, myosin heavy chain (MHC)-isoform distribution; and F, Kv\textsubscript{1.2} in myocyte homogenates from Friend virus B (FVB) and metallothionein (MT) mice with or with buthionine sulfoximine (BSO) treatment. Insets: representative gel blots using respective antibodies. β-actin was used as the loading control. Mean ± SEM, n = 6–8 mice per group. *p < .05 vs. FVB, #p < .05 vs. FVB-BSO.
levels (25). It is not surprising that MT failed to restore the global GSH levels in response to GSH depletion in the current experimental setting. Last, the interrupted balance between the pro- and anti-apoptotic proteins also may contribute to the BSO-induced myocardial defect and MT-elicited cytoprotection.

Our results depict enhanced caspase-3 activity, upregulated pro-apoptotic Bax and p53, elevated phosphorylation of IκB (reduced inhibition of NFκB), and reduced anti-apoptotic Bcl-2 (but not ARC). Potentiation of the oxidative stress response by pro-apoptotic gene expression, formation of p53 fragments, release of cytochrome c, and caspase activation have been established to contribute to deteriorated cardiac function (38). The fact that MT failed to prevent BSO-induced elevated p53 expression indicates a minimal role of p53 in MT-elicited defense under the current experimental setting.
Collectively, data from this study revealed a culprit role of GSH depletion in cardiac functional and ultrastructural abnormalities and, more importantly, the ability of MT to rescue the thiol-rich GSH depletion-induced detrimental myocardial consequences. These results have provided compelling evidence for an essential role of GSH and antioxidants in the maintenance of normal cardiac physiology and management of heart function in critical care medicine.

ACKNOWLEDGMENTS

We thank Dr. D. Paul Thomas, Cindy X. Pang, Janice Audette, and Donna Laturnus for expert technical assistance; and Ms. Virginia L. Cole, University of Wisconsin-Madison for expert technical assistance; X. Fang, Janice Audette, and Donna Laturnus for expert technical assistance; and Ms. Virginia L. Cole, University of Wisconsin-Madison for expert technical assistance.

REFERENCES

17. Li SY, Yang X, Ceylan-Isik AP: Cardiac contractile dysfunction in Lep/Lep obesity is accompanied by NADPH oxidase activation, oxidative modification of sarco(end)oplasmic reticulum Ca2+-ATPase and myosin heavy chain isozyme switch. Diabetologia 2006; 49:1434–1446
37. Lemasters JJ, Qian T, He L: Role of mitochondrial inner membrane permeabilization in necrotic cell death, apoptosis, and autophagy. Antioxid Redox Signal 2002; 4:769–781