Increase in Mrp1 expression and 4-hydroxy-2-nonenal adduction in heart tissue of Adriamycin-treated C57BL/6 mice

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Abstract
Multidrug resistance-associated protein 1 (MRP1) mediates the ATP-dependent efflux of endobiotics and xenobiotics, including estradiol 17-β-D-glucuronide, leukotriene C4, and the reduced glutathione conjugate of 4-hydroxy-2-nonenal (HNE), a highly reactive product of lipid peroxidation. Adriamycin is an effective cancer chemotherapeutic drug whose use is limited by cardiotoxicity. Adriamycin induces oxidative stress and production of HNE in cardiac tissue, which may contribute to cardiomyopathy. We investigated the role of Mrp1 in Adriamycin-induced oxidative stress in cardiac tissue. Mice were treated with Adriamycin (20 mg/kg, i.p.), and heart homogenate and sarcolemma membranes were assayed for Mrp1 expression and ATP-dependent transport activity. Expression of Mrp1 was increased at 6 and 24 hours after Adriamycin treatment compared with saline treatment. HNE-adducted proteins were significantly increased (P < 0.001) in the homogenates at 6 hours after Adriamycin treatment and accumulated further with time; HNE adduction of a 190-kDa protein was evident 3 days after Adriamycin treatment. Mrp1 was localized predominantly in sarcolemma as shown by confocal and Western blot analysis. Sarcolemma membrane vesicles transported leukotriene C4 with a Kₘ and Vₘₐₓ of 51.8 nmol/L and 94.1 pmol/min/mg, respectively, and MK571 (10 μmol/L) inhibited the transport activity by 65%.

Exposure of HEK_Mrp1 membranes to HNE (10 μmol/L) significantly decreased the Vₘₐₓ for estradiol 17-β-D-glucuronide transport by 50%. These results show that expression of Mrp1 in the mouse heart is localized predominantly in sarcolemma. Adriamycin treatment increased Mrp1 expression and HNE adduction of Mrp1. Cardiac Mrp1 may play a role in protecting the heart from Adriamycin-induced cardiomyopathy by effluxing HNE conjugates. [Mol Cancer Ther 2006;5(11):2851–60]

Introduction
Adriamycin is an anthracycline antineoplastic agent that has been commonly used in the United States for treatment of tumor malignancies, including breast cancer, leukemia, and sarcomas. Cardiomyopathy is a major adverse effect in cancer patients who are treated with Adriamycin (1, 2). Patients treated with Adriamycin are 2.5 times more likely to experience cardiomyopathy than untreated patients (3). The incidence of Adriamycin-associated cardiomyopathy and congestive heart failure is dose dependent and varies from 4% at a cumulative dose of 500 to 550 mg/m² to >36% in patients receiving ≥601 mg/m² (4). Several mechanisms have been proposed to explain the etiology of Adriamycin-induced cardiotoxicity, including oxidative damage. Adriamycin induces oxidative stress, lipid peroxidation, and production of lipid aldehydes, including 4-hydroxy-2-nonenal (HNE), in heart tissues as early as 3 hours (5–8). HNE is an α,β-unsaturated aldehyde derived from ω-6 polyunsaturated fatty acids, such as linoleic acid and arachidonic acid (9, 10), and is one of the major and highly toxic products of lipid peroxidation. HNE is a potent electrophile with high reactivity toward cellular nucleophiles; protein residues known to react with HNE are cysteine, histidine, and lysine (9), leading to the hypothesis that these products of lipid peroxidation could play a central role in initiating functional impairment of the myocardium following treatment with Adriamycin. HNE also reacts with intracellular reduced glutathione (GSH) to form GS-HNE (11, 12), which is less toxic than HNE, although it retains some toxicity (13, 14). Thus, metabolic removal of HNE could play an important role in protecting against myocardial injury.

Multidrug resistance-associated protein 1 (MRP1/ABCC1) is a member of the ATP-binding cassette (ABC) transporter protein superfamily, subfamily C (15) and is found predominantly in heart, lung, small intestine, brain, and skin (16, 17). In vitro evidence shows that at least some drugs (e.g., vincristine) are effluxed from cells by MRP1 in the presence of GSH with no molecular modifications (18). MRP1 has also been shown to mediate efflux of sulfate, glucuronide, and glutathione conjugates, such as estradiol...
Adriamycin Increases Mrp1 and HNE in Heart Tissue

Eight- to 12-week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) 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). Mice were treated i.p. with normal saline solution (NSS) or Adriamycin, 20 mg/kg, and heart tissue was examined at various times thereafter as indicated in the figure legends.

**Isolation of Sarcolemmal Membrane**

Heart tissues were dissected and rinsed with isolation buffer consisting of 0.225 mol/L mannitol, 0.075 mol/L sucrose, 1 mmol/L EGTA, and protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin). Tissues were homogenized and centrifuged at 100,000 × g for 40 minutes. Pellets were resuspended in hypotonic lysis buffer [1 mmol/L Tris-HCl, 0.1 mmol/L EDTA (pH 7.4)] containing the protease inhibitors and kept on ice for 40 minutes. Lysed tissue samples were then centrifuged at 100,000 × g at 4°C for 40 minutes, resuspended in TS buffer [10 mmol/L Tris-HCl, 250 mmol/L sucrose (pH 7.4)], and homogenized in a Dounce B tight homogenizer. The homogenate was loaded on 38% (w/v) sucrose in 5 mmol/L Tris-HEPES (pH 7.4) and then centrifuged for 60 minutes at 255,000 × g at 4°C. Membranes were collected from the interface and centrifuged at 100,000 × g for 40 minutes at 4°C. The samples were stored at −80°C until further use.

**Mrp1 Transfection of HEK293**

HEK293 cells were plated and cultured at 37°C in 5% CO2 to reach 80% confluence. Plasmid DNA pCEBV7-Mrp1 (generously provided by Drs. Susan P.C. Cole and Roger G. Deeley, Division of Cancer Biology and Genetics, Cancer Research Institute, Queen’s University, Kingston, Ontario, Canada) was diluted in transfection medium containing TransIT-293 transfection reagent. The DNA-lipid complexes were added to the culture, drop-wise at the side of the flask, and then cultured for 60 to 72 hours at 37°C in 5% CO2. To generate a Mrp1 stable cell line, cells were maintained in culture medium with 50 mg/mL hygromycin B overnight. Medium was replaced every other day with culture medium containing 100 mg/mL hygromycin B for 10 to 14 days or until all cells in the nontransfected control were killed. Subsequently, Mrp1-transfected HEK293 cells (HEKSer) were transferred to a 96-well plate (three–five cells per well) and allowed to grow and form colonies. The colonies surviving in culture medium containing 100 mg/mL hygromycin B were tested for Mrp1 expression by Western blot analysis or immunofluorescent staining and used in the subsequent experiments.

**Isolation of Plasma Membranes from Mrp1-Transfected HEK293 Cells**

HEK293 plasma membranes were isolated as previously described with some modifications (23, 24). In brief, cells were harvested and resuspended in hypotonic lysis buffer with protease inhibitors and kept on ice for 60 minutes. Cell lysates were centrifuged at 100,000 × g at 4°C for 40 minutes, resuspended in Tris-sucrose buffer, and homogenized in a Dounce B tight homogenizer. The homogenate was overlaid on 38% (w/v) sucrose in 5 mmol/L Tris-HEPES (pH 7.4) and centrifuged for 60 minutes at 255,000 × g at 4°C. Membranes were collected from the interface and centrifuged at 10,000 × g for 40 minutes at 4°C. The samples were stored at −80°C until further use.

**Materials and Methods**

**Reagents**

Culture medium and fetal bovine serum were obtained from Life Technologies, Inc. (Rockville, MD). [3H]E217G (39.8 Ci/mmol) and [3H]LTCA (160 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Adriamycin HCl was obtained from Bedford Laboratories (Bedford, OH). TransIT-293 transfection reagent was obtained from Mirus (Madison, WI), and bichoninic acid protein assay reagents were obtained from Bio-Rad Laboratories (Richmond, CA). Rat anti-MRP1 monoclonal antibody was obtained from Alexis (San Diego, CA), Alexa Fluor 488 donkey anti-rat IgG was obtained from Molecular Probes (Eugene, OR), rabbit anti-MRP1-Michael adduct polyclonal antibody was purchased from Calbiochem (San Diego, CA), mouse anti-Na+/K+-ATPase α1 monoclonal antibody was purchased from Upstate (Lake Placid, NY), mouse anti-endothelial nitric oxide synthase (eNOS) antibody was purchased from BD Transduction (San Diego, CA), and goat anti-mouse Cy5 was obtained from Zymed (South San Francisco, CA). Anti-rat Ig-horseradish peroxidase (HRP), anti-rabbit Ig-HRP, anti-mouse Ig-HRP, and Enhanced Chemiluminescence Plus were obtained from Amersham Biosciences (Piscataway, NJ).

**Animals**

Eight- to 12-week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) 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). Mice were treated i.p. with normal saline solution (NSS) or Adriamycin, 20 mg/kg, and heart tissue was examined at various times thereafter as indicated in the figure legends.
Na\textsuperscript{+}/K\textsuperscript{+}-ATPase Activity Assay

The ATPase activity assay was done as described (25). Briefly, 970 \muL of reaction buffer containing 125 mmol/L Tris, 1 mmol/L EGTA, 120 mmol/L NaCl, 12.5 mmol/L KCl, 5 mmol/L Na2HPO4, 1.7 mmol/L NaH2PO4, 5 mmol/L MgCl2, 5 mmol/L ATP, 2.5 mmol/L phosphoenolpyruvate, and 0.5 mmol/L NAD were prewarmed for 3 minutes at 37°C in a temperature-controlled cuvette compartment of a UV spectrophotometer (Shimadzu UV-1601, Shimadzu Scientific Instruments, Columbia, MD). Subsequently, 10 \muL of lactic dehydrogenase and pyruvate kinase (10 units/mL) were added followed by 20 \muL of tissue samples (10 \mug/\muL each, in the presence or absence of 2 mmol/L ouabain, were added followed by 20 \muL of tissue samples (10 \mug/\muL each). Oxidation of NADH was continuously monitored at 340 nm. ATPase activity was calculated from the slope of the linear portion of the reaction, the NADH millimolar extinction coefficient, and the volume of the reaction mixture and expressed per milligram of protein.

Immunoprecipitation

Total heart homogenate (300 \mug) was resuspended in 150 \muL of radioimmunoprecipitation assay buffer (9.1 mmol/L Na2HPO4, 1.7 mmol/L NaH2PO4, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% (v/v) NP40 (pH 7.2)). Anti-MRP1 monoclonal antibody (3 \mug) was added and incubated overnight at 4°C. Protein A/G agarose (30 \muL; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the mixture and incubated overnight. Immunocomplexes were collected by centrifugation at 600 \times g for 5 minutes at 4°C followed by washing with radioimmunoprecipitation assay buffer, four times. Immunoprecipitated samples were recovered by resuspending them in 2 \times sample loading buffer, boiled for 5 minutes, and were fractionated by 4% to 12% Tris-glycine SDS-PAGE (Invitrogen, Carlsbad, CA) and analyzed by Western blot.

Immunoblot Assay

The protein concentrations were determined with the bicinchoninic acid protein assay using bovine serum albumin as standard. Protein samples were fractionated on a 4% to 12% SDS-PAGE gel and transferred to nitrocellulose (Whatman, Inc., Stanford, ME). The blots were incubated with the primary antibody (1:1,000 rat anti-MRP1 monoclonal antibody, 1:500 rabbit anti-HNE-Michael adduct polyclonal antibody, 1:1,000 anti-Na\textsuperscript{+}/K\textsuperscript{+}-ATPase \alpha1, and 1:1,000 anti-eNOS) diluted in TBS containing 5% nonfat milk and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma-Aldridge, St. Louis, MO), washed in TBS/0.1% Tween 20, and subsequently incubated with HRP-labeled secondary antibody (1:5,000 anti-rat IgG-HRP, 1:5,000 anti-mouse IgG-HRP, and 1:4,000 anti-rabbit IgG-HRP) in TBS/5% nonfat milk/0.1% Tween 20. Chemiluminescence detection was done using Enhanced Chemiluminescence Plus and exposure to BioMax MR film (Kodak, Rochester, NY).

Confocal Scanning Laser Microscopy

Heart samples were snap frozen in 2-methylbutane precooled in liquid nitrogen and stored at −80°C. Cryosections (4 \muM) of hearts (left ventricle) were prepared with a Zeiss Microm HM5000 microtome cryostat (Carl Zeiss, Inc., Thornwood, NY). Tissue sections were air dried for 2 hours and fixed in methanol at −20°C for 10 minutes. Tissue sections were blocked with 2% bovine serum albumin for 60 minutes at room temperature and incubated with primary mouse anti-Na+/K+-ATPase \alpha1 antibody (1:200) or rat anti-MRP1 antibody (1:100) at 4°C overnight. Tissue sections were then washed and probed with goat anti-mouse Cy5 (1:200) or Alexa Fluor 488 donkey anti-rat (1:200) antibodies at room temperature for 2 hours in the dark. Tissue sections were washed and air dried, then mounting medium was added, and sections were placed under a cover glass.

Nuclei of HEK293 and Mrp1-transfected HEK293 (HEKMrp1) cells were stained with Hoechst 33342. Cells were washed with PBS, fixed with 4% paraformaldehyde, and then blocked with 1% bovine serum albumin for 10 minutes at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes and stained with rat anti-MRP1 antibody (1:2,500), washed, and subsequently incubated with Alexa Fluor 488 donkey anti-rat IgG (1:1,000).

Images were taken with a confocal scanning laser microscope (True Confocal Scanner 4D, Leica, Heidelberg, Germany) equipped with two photon IR lasers as well as an argon laser (green fluorescence), krypton laser (red fluorescence), helium-neon laser (far-red fluorescence), and a transmitted light detector for differential interference contrast and phase microscopy.

Transport Studies

Membranes isolated from HEK293, HEKMrp1, or sarcolemmamembranes were incubated with DMSO, 1 or 10 \mumol/L HNE in DMSO (4%, final concentration) for 20 minutes at 37°C. Before each membrane incubation, the filtration cylinder and filters were rinsed with 1 mmol/L sulfobromophthalein to reduce nonspecific binding of [3H]E217G. ATP-dependent transport into membrane vesicles was determined as described (26) in a Tris-sucrose buffer containing 5 mmol/L ATP or AMP, 10 mmol/L MgCl2, 10 mmol/L phosphocreatine, 100 \mug/mL creatine phosphokinase, and 10 \mumol/L [3H]E217G in DMSO (4%, final concentration). ATP-dependent transport of [3H]E217G into membrane vesicles (4 \mug protein/20 \muL) was measured in incubations at 37°C for 1 minute or as indicated in figure legends, transport was stopped with 3.5 mL of ice-cold stop buffer, and the mixture was quickly filtered onto a Durapore 0.45-\muM HV filter (Millipore Corp., Bedford, MA).

ATP-dependent transport of [3H]LTC4 was assayed in HEK or sarcolemma membranes and carried out at 23°C in an incubation buffer as described above in the presence or absence of MK571 (10 \mumol/L). The concentrations of [3H]LTC4 ranged from 10 to 1,000 nmol/mL (25 nCi) as indicated in figure legends. The reaction was stopped and filtered onto a glass fiber filter (GF/F, Whatman, Inc., Clifton, NJ) as described for E217G.

[3H]LTC4 collected on the filters was detected by liquid scintillation counting using scintillation counting cocktail (Bio-Safe II, Research Products International Corp., Mt. Prospect, IL).
Statistical Analysis

Data of quantitative results were expressed as mean ± SE or as otherwise indicated. Statistical analyses were done using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). In multiple comparisons, one-way ANOVA followed by a post hoc test was used. P < 0.05 was considered a significant difference. Calculations of kinetic variables were carried out by nonlinear regression analysis fitted to the Michaelis-Menten equation using GraphPad Prism 4.

Results

Adriamycin Increases Mrp1 Protein Levels in Heart Tissue

To examine the effect of Adriamycin on expression of Mrp1, C57BL/6 mice were treated with saline or Adriamycin and heart homogenates were prepared. Western blot analysis of heart homogenate of saline controls indicated that Mrp1 was constitutively present in heart tissue and that Mrp1 expression was significantly increased 1.3-fold (6 hours) and 1.2-fold (24 hours) compared with saline control following Adriamycin treatment (Fig. 1). eNOS was used as a loading control, as the expression of eNOS does not change with Adriamycin treatment (27). These data indicated that Adriamycin enhanced Mrp1 expression in cardiac tissue.

Mrp1 Is Localized in Sarcolemmal Membrane

Confocal immunofluorescent microscopy was used to localize the expression of Mrp1 in cardiac tissue and showed that Mrp1 is constitutively expressed in cardiomyocytes predominantly in the sarcolemma (Fig. 2A). On Adriamycin treatment, Mrp1 expression was increased and colocalized with Na⁺/K⁺-ATPase in the sarcolemma (Fig. 2B). To further characterize the localization of Mrp1, sarcolemmal membranes were isolated by sucrose gradient assay and assayed for Mrp1 and Na⁺/K⁺-ATPase α1 by immunoblot 3 days after Adriamycin treatment. Mrp1 protein expression in sarcolemma was greatly enriched versus that in whole-heart homogenate (Fig. 2C). A similar membrane was probed for Na⁺/K⁺-ATPase α1 and showed that Na⁺/K⁺-ATPase was similarly highly enriched in the sarcolemma fraction compared with heart homogenate (Fig. 2D).

We also characterized ATP-dependent transport activity in sarcolemmal membrane. As shown in Fig. 3A, Mrp1 was highly expressed in the plasma membranes of HEK293 cells transfected with Mrp1 (HEK<sub>Mrp1</sub>). Sarcolemma membrane vesicles were capable of transporting E<sub>2</sub>17G almost as efficiently as HEK<sub>Mrp1</sub> plasma membrane vesicles (Fig. 3B). We also characterized LTC4 transport in sarcolemma because this substrate is more specific for Mrp1 versus other ATP-dependent transports known to be expressed in cardiac tissue, such as P-glycoprotein (28, 29). The kinetic variables of LTC4 transport of sarcolemma membranes were determined (Fig. 3C), and nonlinear regression analysis of the data yielded K<sub>m</sub> and V<sub>max</sub> values of 51.8 nmol/L and 94.1 pmol/min/mg, respectively (Table 1). In the presence of the MRP inhibitor MK571 (10 μmol/L), ATP-dependent transport activity in sarcolemma vesicles from Adriamycin-treated mice (1 day) was inhibited by 65% (Fig. 3D), further supporting the role of Mrp1 in mediating the ATP-dependent transport in sarcolemma. These results clearly show that Mrp1 is localized predominately in the sarcolemmal membranes and functions as an efflux pump in cardiomyocytes.

Time Course for Appearance of Adriamycin-Induced HNE Adduction of Proteins in Heart

We investigated the effect of Adriamycin treatment on the adduction of heart proteins with the highly reactive product of lipid peroxidation, HNE, using an antibody that recognizes Michael adducts of HNE (30, 31). Mice were treated with saline or Adriamycin, and cardiac tissue was examined for HNE-protein adduction at the indicated times thereafter. The levels of HNE adduction were normalized to that of saline controls. HNE adduction of proteins was significantly increased as early as 6 hours after Adriamycin treatment. At 72 hours after Adriamycin, HNE adduction was 1.5-fold higher relative to saline-treated mice (Fig. 4A).

We also questioned whether HNE adduction of Mrp1 might occur in Adriamycin-treated heart; consequently, we assayed heart homogenate for HNE-Michael adducts by Western blot analysis. There was a marked increase in HNE adduction of a 190-kDa protein from heart homogenates 3 days after treatment with Adriamycin compared with the saline control (Fig. 4B). To determine if HNE adduction of the protein (molecular weight, 190 kDa) was indeed associated with Mrp1, we isolated plasma membranes.
from HEKMrp1 to use as a Mrp1-positive control protein. When the membrane was reprobed with anti-MRP1 antibody, the band of protein (190 kDa) specific to Mrp1 as confirmed by the HEKMrp1 membrane was shown to be increased in Adriamycin-treated mice (Fig. 4C). To confirm that the 190-kDa adducted protein was indeed Mrp1, total heart homogenate was immunoprecipitated with MRP1 antibody and the precipitate was analyzed for HNE-Michael adducts by Western blotting. As shown in Fig. 4D, HNE was coprecipitated with Mrp1 protein following Adriamycin treatment. These results indicated that Adriamycin markedly increased HNE-protein adducts in cardiac tissue, including adduction of Mrp1.

**Inhibition of Transport of [3H]E217G by HNE**

To determine if HNE-protein adduction in heart might interfere with the functional properties of Mrp1, we exposed HEKMrp1 membranes to HNE (1 and 10 μmol/L) or DMSO alone at 37°C for 20 minutes and characterized ATP-dependent transport of [3H]E217G. As shown in Fig. 5, incubation of HEKMrp1 membranes with HNE at 37°C for 20 minutes inhibited ATP-dependent transport in a dose-response manner. The transport activity of Mrp1 was significantly reduced from 49.7 in DMSO-treated membranes to 31.4 (P < 0.05) and 28.1 pmol/min/mg protein (P < 0.01) in membranes treated with 1 and 10 μmol/L HNE, respectively.

To investigate whether HNE might affect kinetics of Mrp1 transport, a time course of [3H]E217G uptake was determined. The uptake of [3H]E217G in HEKMrp1 membranes treated with 10 μmol/L HNE at 1, 3, and 5 minutes was decreased significantly (Fig. 6A). The kinetic variables of E217G transport in DMSO versus 10 μmol/L HNE-treated HEKMrp1 membranes were determined (Fig. 6B). Nonlinear regression analysis of the data yielded Km and Vmax values (Table 2) showing that HNE treatment had minimal effects on the Km value (7 versus 5 μmol/L) but decreased the Vmax by ~50%, from 54.3 to 26.9 pmol/min/mg protein. These data indicated that HNE is a potent inhibitor of Mrp1 function.

**Discussion**

Anthracyclines are classified as antitumor antibiotics and play a pivotal role in the treatment of both solid and hematologic malignancies. However, their clinical use is limited by dose-dependent cardiotoxic side effects (3). Anthracyclines, particularly Adriamycin, contain a quinone functional group that can undergo redox cycling, generating free radicals, including reactive oxygen species that are associated with subsequent cardiac toxicity, as evidenced by vacuolar degeneration of the sarcoplasmic reticulum (32, 33). A major product of the free radicals generated by oxidative stress includes the α,β-unsaturated aldehydes, which are produced during β-scission of alkoxyl radicals derived from α-6 polyunsaturated fatty acids (9, 10, 30, 31). These aldehydes are highly reactive and are generated in high concentrations by peroxidative reactions of lipids and lipoproteins. Toxicity due to α,β-unsaturated aldehydes is considered a major component of tissue injury resulting from reactive oxygen species (9, 10). HNE is a potent electrophile and is one of the most toxic aldehydes generated during lipid peroxidation (9, 10, 30, 31). HNE...
is partially detoxified by conjugation with GSH by glutathione S-transferases (34), which are present in cardiac tissue (35). Perfusion of the heart with HNE depletes intracellular GSH accompanied by an increase in GS-HNE that is effluxed into the perfusion medium by a saturable process (35). Ishikawa (36) further showed that rat heart sarcolemma vesicles transport the glutathione conjugate S-(2,4-dinitrophenyl)-glutathione, now recognized as a classic Mrp1 substrate, in an ATP-dependent and saturable manner and that this transport was inhibited by GS-HNE. Srivastava et al. (37) later characterized the metabolism of HNE by the isolated perfused rat heart and showed the formation and release of GS-HNE. These data suggest an important role for GSH in protecting the heart, as it not only conjugates with HNE to decrease its chemical reactivity but also forms a Mrp1 substrate, which in turn is effluxed from the tissue. Recent studies (14) have shown that MRP1 mediates the ATP-dependent transport of [3H]GS-HNE. Taken together, these data strongly implicate Mrp1 in mediating the efflux of GS-HNE from the heart.

Table 1. Kinetics of [3H]LTC4 transport by sarcolemma

<table>
<thead>
<tr>
<th>Membrane</th>
<th>$K_m$ (nmol/L)</th>
<th>95% CI</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEKMrp1</td>
<td>73.0 ± 20.2</td>
<td>16.9-129.0</td>
<td>185.4 ± 13.42</td>
<td>148.1-222.6</td>
</tr>
<tr>
<td>Sarcolemma</td>
<td>51.8 ± 20.1</td>
<td>0-107.6</td>
<td>94.1 ± 8.5</td>
<td>70.4-117.8</td>
</tr>
</tbody>
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NOTE: HEK293 membranes expressing Mrp1 (HEKMrp1) or sarcolemma membranes from NSS-treated mice were incubated with [3H]LTC4 at 23°C for 1 minute. The initial rate of ATP-dependent [3H]LTC4 uptake was measured at various LTC4 concentrations (10–1,000 nmol/L), and $K_m$ and $V_{max}$ were determined. Data represent the mean ± SD from triplicate determinations.

Abbreviation: 95% CI, 95% confidence interval.
We therefore characterized Mrp1 expression and activity in mouse heart and the effects of Adriamycin treatment on its expression. The present studies clearly show expression and transport activity of Mrp1 in mouse sarcolemma, that expression of Mrp1 increases following Adriamycin treatment, and that Adriamycin treatment leads to adduction of Mrp1 with HNE.

In vitro studies using HEK293 cells overexpressing Mrp1 further showed that exposure to HNE decreases Mrp1 transport activity. It is important to note that, in these studies, we took advantage of mouse Mrp1 to study its role in heart. Whereas the human isoform (MRP1) is able to transport natural product-type drugs (e.g., the anthracyclines, epipodophyllotoxins, and Vinca alkaloids in conjunction with GSH), murine Mrp1 confers negligible resistance to anthracycline antibiotics, such as doxorubicin, daunorubicin, and epirubicin (38). MRP1/Mrp1 chimeras have shown that this difference is due to two amino acid differences in the COOH-terminal region of the protein (39, 40). Thus, any effects of Mrp1 in the present studies cannot be attributed to its role in the cellular efflux of Adriamycin.

MRP1 is ubiquitously expressed, with relatively high expression in the heart. Flens et al. (16) first reported the presence of MRP1 in normal myocardium; however, the present study is the first to identify the expression of Mrp1 in mouse heart and that Mrp1 is predominantly localized in the sarcolemma (Fig. 2). Taken together with data described above showing the saturable efflux of GSH conjugates from the heart, the presence of Mrp1 in the sarcolemma implies that Mrp1 plays a pivotal physiologic role in protecting cardiomyocytes from oxidative damage. In fact, Ishikawa and Sies (41) had noted the substantially lower capacity of heart for oxidized glutathione transport and reduction compared with liver, explaining, in part, the particular sensitivity of cardiac tissue to oxidative damage. Recently, it has been shown that MRP1 is the main transporter protein that effluxes oxidized glutathione from endothelial aorta (42), thus supporting the role of MRP1 in modulating oxidative stress in the cardiovascular system.

Several ABC transport mRNA/proteins have been reported to be present in heart to various degrees (43). Although its substrate specificity overlaps with that of MRP1 (44), there is no/very low expression level of MRP2 in heart. Compared with MRP1, MRP3 is a low-affinity glutathione conjugate transporter (45, 46). MRP4 mRNA is intermediately expressed in heart (43); however, there is no evidence of MRP4 protein expression in heart tissue. In addition, MRP4 has a low affinity for E217G (Km = 30.3 μmol/L), and there are no reports of MRP4-mediated LTC4 transport (47, 48). Similarly, Bcrp1/ABCG2 is expressed in heart; however, the affinity of ABCG2 for E217G is 44.2 μmol/L (49), 10-fold higher than the values reported for Mrp1 (4.8 μmol/L; ref. 38), suggesting an apparently limited contribution of ABCG2 to E217G transport. In addition, LTC4 (2 μmol/L) did not inhibit transport of [3H]estrone 3-sulfate ([3H]E1S) in vesicles prepared from ABCG2-transfected P388 cells (50), indicating that LTC4 is a poor substrate for ABCG2. Finally, MK571 (10 μmol/L),
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Figure 6. Inhibition of \(^{3}H\)E217G transport by HNE. HEK membranes expressing Mrp1 (HEKMrp1) were incubated with 10 \(\mu\)mol/L HNE in DMSO at 37°C for 20 min, and ATP-dependent transport was determined. A, \(^{3}H\)E217G uptake was measured in membrane vesicles prepared from HEK (○), HEKMrp1/DMSO (■), and HEKMrp1/HNE (10 \(\mu\)mol/L; □). Points, mean of two independent experiments; bars, SE. *, \(P < 0.05\) versus DMSO-treated HEKMrp1; ***, \(P < 0.001\) versus DMSO-treated HEKMrp1. B, kinetics of ATP-dependent E217G uptake by HEKMrp1/DMSO (○) and HEKMrp1/HNE (10 \(\mu\)mol/L; □). The initial rate of ATP-dependent \(^{3}H\)E217G uptake was measured at various E217G concentrations (0.1–15 \(\mu\)mol/L) for 1 min at 37°C as described above. Points, mean of three independent experiments; bars, SE.

A MRP-specific inhibitor, was an effective inhibitor of LTC4 transport in sarcolemma vesicles. Taken together, these data indicate that Mrp1 is the major protein in cardiac tissue mediating the transport of these organic anion conjugates.

We found that Mrp1 was constitutively expressed in heart tissue at low levels and that its expression was increased after Adriamycin treatment (Fig. 1). Exposure of GLC4 cells to Adriamycin in vitro has been shown to induce Mrp1 mRNA expression (51), suggesting a similar mechanism in the present studies. Although the current studies were not designed to identify the mechanism of increased Mrp1 expression, the presence of an antioxidant response element/activator protein-1 binding site in the Mrp1 promoter (52) suggests that Adriamycin-induced oxidative stress may contribute to increased expression of Mrp1 in the heart. Indeed, Adriamycin leads to increased expression of Mrp1 in mouse brain, in which elevated oxidative stress is observed (53). Mrp1 is also elevated in Alzheimer’s disease brain (31), which is under extensive oxidative stress (54). The mechanism by which Adriamycin increases Mrp1 protein expression in heart is currently under investigation in our laboratory.

Total HNE-protein adduction also accumulated with time and peaked at 72 hours after Adriamycin treatment (Fig. 4A). Similarly, there was an increase in a HNE adduct of a 190-kDa protein in heart that migrated with Mrp1 following Adriamycin treatment. Under Adriamycin-induced oxidative stress conditions where lipid peroxidation occurs, HNE is generated, leading to HNE adduction and, possibly, cross-linking of membrane proteins (55). Oxidized and cross-linked proteins are less susceptible than native proteins to proteolysis such that oxidation prevents their efficient degradation, leading to the persistence of oxidized protein in tissue (10, 56). These data are consistent with the accumulation of HNE adduction found in heart tissue (Fig. 4A) that could result in inactivation of Mrp1 and prevent it from effluxing GS-HNE. Renes et al. (14) showed that inhibition of GS-HNE efflux from cells with MK571, a MRPI inhibitor, increased the toxicity of HNE to MRPI-expressing cells, supporting the hypothesis that extrusion of GS-HNE from cells is required to prevent toxicity (31). As noted previously, lipid-derived alkenals, such as HNE, are found in cardiac tissue following Adriamycin treatment and are thought to contribute to Adriamycin-induced cardiomyopathy (6). These data suggest that HNE adduction of Mrp1 and inhibition of its transport activity could further increase toxicity and damage to the heart.

We examined the effect of HNE on Mrp1 function using plasma membranes from HEK293 cells that overexpressed Mrp1. Incubation of HEKMrp1 membranes with HNE at levels that are consistent with those generated in vivo under oxidative stress conditions (57) significantly decreased membrane transport activity by ~40% (Figs. 5 and 6). Taken together, these results suggest that the up-regulation of Mrp1 in cardiac tissue following Adriamycin treatment has physiologic relevance in protecting the heart by mediating the efflux of toxic products of oxidative stress. However, adduction of Mrp1 with HNE under conditions of high oxidative stress likely causes dysfunction of Mrp1 and may lead to further cardiac injury. These data suggest that inhibition of Mrp1 may contribute to Adriamycin-induced cardiomyopathy. Further in vitro and in vivo investigations will be necessary to substantiate this hypothesis.
One of the strategies in treatment of resistant cancer is to inhibit ABC transporter proteins. The rationale behind this thought is to maintain cytotoxic drug concentrations within cancer cells. Several cancer cells overexpress MRP1 and are thereby resistant to standard chemotherapy (58, 59). Inhibition of MRP1 is therefore thought likely to increase the success of cancer treatment. However, because MRP1 is ubiquitously expressed in several organs (16), it also possible that use of a MRP1 inhibitor could cause toxicity to normal tissues by blocking the normal protective function of MRP1, particularly in tissues, such as the heart, which are highly susceptible to oxidative injury. Recently, Wojnowski et al. (60) showed that single-nucleotide polymorphisms of MRP1 in non-Hodgkin’s lymphoma patients are associated with Adriamycin-induced cardiotoxicity. It will be important to determine if such single-nucleotide polymorphisms influence MRP1-mediated transport of Adriamycin and/or GS-HNE to contribute to Adriamycin-induced cardiotoxicity.

In summary, we showed that sarcolemma constitutively expressed Mrp1. Adriamycin treatment increased Mrp1 expression, particularly in sarcolemma, concurrent with increased HNE-protein adduction in cardiac tissue. Sarcolemma membrane vesicles efficiently transported E217G and LTC4, and HNE significantly inhibited Mrp1 activity. These data suggest that a physiologic function of Mrp1 in heart is to protect cardiomyocytes from toxic products of lipid peroxidation, such as GS-HNE, a product of oxidative stress in the heart.

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Molecular Cancer Therapeutics

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