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-(3-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 lipid peroxidation. Adriamycin-induced 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 predominately 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%.

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...
17-(β-d-glucuronide) (E217G) and cysteinyl leukotriene C₄ (LTC₄; refs. 14, 19). Importantly, MRPI expressed in SF9 cells has been shown to mediate the transport of GS-HNE (apparent Kᵅᵅ = 1.6 µmol/L; ref. 14). Although Mrp1-null mice have no phenotype and have normal fertility and viability, their ability to transport some endobiotics and xenobiotics is compromised. Mrp1⁻/⁻ mice are hypersensitive to some anticancer drugs, such as etoposide (20), and an impairment in the efflux of [¹⁴C]grepafloxacin and Fluor3 in the skin (21). In addition, mice lacking Mrp1 have a transiently impaired immune response during tuberculosis (22). These data suggest that Mrp1 plays a significant role in the efflux of endobiotics and xenobiotics and their conjugates from various organs and may protect tissues from toxicity, including that induced by lipid peroxidation.

In this study, we investigated the role of MRP1 in cardiac tissue and questioned whether it might be important in protecting the heart against Adriamycin-induced cardiac toxicity. We found that Adriamycin increased expression of Mrp1 in cardiac tissue. In addition, HNE adduction of Mrp1 in heart tissue was also increased by Adriamycin treatment. Using Mrp1-transfected HEK293 cells, we showed that HNE inhibited Mrp1 transport function.

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]LTC₄ (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 bichinonic acid protein assay reagents were obtained from Bio-Rad (Richmond, VA). 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.

Isolation of Sarcoplemmal 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 aprotonin, 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 overlaid on 38% (w/v) sucrose in 5 mmol/L Tris-HCl, 250 mmol/L sucrose (pH 7.4), and centrifuged for 40 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 F.C. Cole and Roger G. Deely, 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 (HEK₅Mrp1) 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 100,000 × g for 40 minutes at 4°C. The samples were stored at −80°C until further use.
Na\(^+/K\(^+-\)ATPase Activity Assay

The ATPase activity assay was done as described (25). Briefly, 970 \(\mu\)L of reaction buffer containing 125 mmol/L Tris, 1 mmol/L EGTA, 120 mmol/L NaCl, 12.5 mmol/L KCl, 5 mmol/L Na\(_2\)HPO\(_4\), 1.7 mmol/L NaH\(_2\)PO\(_4\), 150 mmol/L 150 L of tissue samples (10 \(\mu\)g/\(\mu\)L), were added followed by 20 \(\mu\)L of DMSO, 1 or 10 \(\mu\)mol/L HNE in DMSO (4%, final concentration) for 20 minutes at 37°C. Before each membrane incubation, the filtration cylinder was first washed with 1 mmol/L sulfobromophthalein to reduce nonspecific binding of [3H]E217G. Membrane homogenates were incubated with DMSO, 1 or 10 \(\mu\)mol/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 of [3H]E217G into membrane vesicles was determined as described (26) in a Tris-sucrose buffer containing 5% nonfat milk and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma-Aldrich, St. Louis, MO), washed in TBS/0.1% Tween 20, and subsequently incubated with HRP-labeled secondary antibody (1:5,000 anti-rat Ig-HRP, 1:5,000 anti-mouse Ig-HRP, and 1:4,000 anti-rabbit Ig-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 \(\mu\)m) 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 α1 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 coverslip.

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, HEK<sub>Mrp1</sub>, or sarcolemma were incubated with DMSO, 1 or 10 \(\mu\)mol/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]LTC4. 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 MgCl\(_2\), 10 mmol/L phosphocreatine, 100 \(\mu\)g/mL creatine phosphokinase, and 10 mmol/L [3H]E217G in DMSO (4%, final concentration). ATP-dependent transport of [3H]E217G into membrane vesicles (4 \(\mu\)g protein/20 \(\mu\)L) 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-\(\mu\)m 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 \(\mu\)mol/L). The concentrations of [3H]LTC4 ranged from 10 to 1,000 nmol/L (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).
**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 and assayed for Mrp1 and Na\(^+/\)K\(^{-}\)-ATPase \(\alpha1\) 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 \(\alpha1\) 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\(_{\text{Mrp1}}\)). Sarcolemma membrane vesicles were capable of transporting E\(_2\)17G almost as efficiently as HEK\(_{\text{Mrp1}}\) plasma membrane vesicles (Fig. 3B). We also characterized LTC\(_4\) 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 LTC\(_4\) transport of sarcolemma membranes were determined (Fig. 3C), and nonlinear regression analysis of the data yielded \(K_m\) and \(V_{\text{max}}\) values of 51.8 nmol/L and 94.1 pmol/min/mg, respectively (Table 1). In the presence of the MRP inhibitor MK571 (10 \(\mu\)mol/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 predominantly 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.

**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.
from HEK_{Mrp1} 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 HEK_{Mrp1} 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 \[^{3}H\]E217G by HNE**

To determine if HNE-protein adduction in heart might interfere with the functional properties of Mrp1, we exposed HEK_{Mrp1} membranes to HNE (1 and 10 \(\mu\)mol/L) or DMSO alone at 37°C for 20 minutes and characterized ATP-dependent transport of \[^{3}H\]E217G. As shown in Fig. 5, incubation of HEK_{Mrp1} 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 \(\mu\)mol/L HNE, respectively.

To investigate whether HNE might affect kinetics of Mrp1 transport, a time course of \[^{3}H\]E217G uptake was determined. The uptake of \[^{3}H\]E217G in HEK_{Mrp1} membranes treated with 10 \(\mu\)mol/L HNE at 1, 3, and 5 minutes was decreased significantly (Fig. 6A). The kinetic variables of E217G transport in DMSO versus 10 \(\mu\)mol/L HNE-treated HEK_{Mrp1} membranes were determined (Fig. 6B). Nonlinear regression analysis of the data yielded \(K_m\) and \(V_{max}\) values (Table 2) showing that HNE treatment had minimal effects on the \(K_m\) value (7 versus 5 \(\mu\)mol/L) but decreased the \(V_{max}\) 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 \(\alpha,\beta\)-unsaturated aldehydes, which are produced during \(\beta\)-scission of alkoxyl radicals derived from \(\omega\)-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 \(\alpha,\beta\)-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).
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.

Figure 3. Mrp1-mediated transport of E217G and LTC4 in HEKMrp1 and sarcolemmal membrane. A, HEK293 cells were transfected with pCEBV7-Mrp1 plasmid using TransIT-293 reagent. Cells were fixed and stained with anti-Mrp1 antibody and visualized with anti-rat Alexa Fluor 488 fluorescent antibody (green) under confocal microscope. Blue, nuclei were counterstained with Hoechst 33342. Plasma membranes of HEK293 cells were isolated and kept at −80°C for the subsequent experiments. B, ATP-dependent transport of [3H]E217G (10 μmol/L) into sarcolemmal membrane vesicles (10 μg/20 μL) was measured at 37°C for 1 min. HEK-transfected Mrp1 (HEKMrp1) membranes were used as a positive control. Columns, mean of triplicate determinations; bars, SD. C, HEKMrp1 (○) or sarcolemma membranes of NSS-treated mice ( ● ) were incubated at 23°C for 1 min, and kinetics of ATP-dependent [3H]LTC4 were determined. The initial rate of ATP-dependent [3H]LTC4 uptake was measured at various [3H]LTC4 concentrations (10−1,000 nmol/L). Points, mean of triplicate determinations; bars, SD. D, HEK (white columns) or sarcolemma membranes (black columns) were incubated with 100 nmol/L LTC4 (25 nCi) in the presence or absence of MK571 (10 μmol/L) at 23°C for 1 min, and ATP-dependent transport was determined. Columns, mean of two independent experiments; bars, SE. **, P < 0.01 versus untreated sarcolemma; ***, P < 0.001 versus untreated HEKMrp1. HEK, nontransfected HEK293 cells; NSS, sarcolemma isolated from saline-treated mice.

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 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 MR2 in heart. There is also very low to no expression of MR3 in heart. Compared with MRP1, MR3 is a low-affinity glutathione conjugate transporter (45, 46). MR4 mRNA is intermittently 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),

![Figure 4](image_url)  
**Figure 4.** HNE-adducted proteins and Mrp1 protein levels in cardiac tissue from Adriamycin versus saline-treated mice. A, mice were treated with saline control or Adriamycin (20 mg/kg, i.p.), and cardiac tissue was examined at the indicated times thereafter. **, P < 0.01; ***, P < 0.001. B, Western blot of HNE-Michael adducts at 72 h was confirmed and showed a HNE adduct at 190 kDa. C, the blot was stripped and reprobed for Mrp1. HEK293-transfected Mrp1 (HEKmp1) membranes were used as a positive control. D, samples of whole-heart homogenate were immunoprecipitated with Mrp1 antibody and analyzed for levels of HNE-Michael adducts by Western blotting. Pellet, immunoprecipitated protein; Sup, supernatant following immunoprecipitation.

![Figure 5](image_url)  
**Figure 5.** Inhibition of [3H]E217G transport by HNE. HEK membranes expressing Mrp1 (HEKmp1) were incubated with the indicated concentrations of HNE in DMSO at 37°C for 20 min, and ATP-dependent transport over 1 min was determined. Columns, mean of three independent experiments; bars, SE. *, P < 0.05 versus DMSO-treated HEKmp1; **, P < 0.01 versus DMSO-treated HEKmp1.
Adriamycin Increases Mrp1 and HNE in Heart Tissue

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 conditions (57) significantly decreased 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.

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, addition 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(K_m) ((\mu\text{mol/L}))</th>
<th>95% CI</th>
<th>(V_{\text{max}}) (pmol/min/mg)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>7.0 ± 2.9</td>
<td>0.14-15</td>
<td>54.3 ± 10.2</td>
<td>28.1-80.6</td>
</tr>
<tr>
<td>HNE</td>
<td>5.0 ± 2.4</td>
<td>0.11-12</td>
<td>26.9 ± 5.2</td>
<td>13.6-40.2</td>
</tr>
</tbody>
</table>

NOTE: HEK293 membranes expressing Mrp1 (HEKMrp1) were incubated with 10 \(\mu\text{mol/L}\) HNE in DMSO at 37°C for 20 minutes. The initial rate of ATP-dependent \([\text{H}]\text{E}_{217}\text{G}\) uptake was measured at various \(\text{E}_{217}\text{G}\) concentrations (0.1–15 \(\mu\text{mol/L}\)) for 1 min at 37°C as described above. Points, mean of three independent experiments; bars, SE.

Figure 6. Inhibition of \([\text{H}]\text{E}_{217}\text{G}\) transport by HNE. HEK membranes expressing Mrp1 (HEKMrp1) were incubated with 10 \(\mu\text{mol/L}\) HNE in DMSO at 37°C for 20 min, and ATP-dependent transport was determined. A, \([\text{H}]\text{E}_{217}\text{G}\) uptake was measured in membrane vesicles prepared from HEK (○), HEKMrp1/DMSO (●), and HEKMrp1/HNE (10 \(\mu\text{mol/L};\) ◊). Points, mean of two independent experiments; bars, SE. **, \(P < 0.001\) versus DMSO-treated HEKMrp1. B, kinetics of ATP-dependent \(\text{E}_{217}\text{G}\) uptake by HEKMrp1/DMSO (●) and HEKMrp1/HNE (10 \(\mu\text{mol/L};\) ◊). The initial rate of ATP-dependent \([\text{H}]\text{E}_{217}\text{G}\) uptake was measured at various \(\text{E}_{217}\text{G}\) concentrations (0.1–15 \(\mu\text{mol/L}\)) for 1 min at 37°C as described above. Points, mean of three independent experiments; bars, SE.

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 MRP inhibitor, increased the toxicity of HNE to MR1-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.
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 sarcosome constitutively expressed Mrp1. Adriamycin treatment increased Mrp1 expression, particularly in sarcosome, concurrent with increased HNE-protein adduction in cardiac tissue. Sarcosome membrane vesicles efficiently transported E2LG 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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Adriamycin Increases Mrp1 and HNE in Heart Tissue


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

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