Exposing cardiomyocytes to subclinical concentrations of doxorubicin rapidly reduces their creatine transport

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Submitted 8 February 2012; accepted in final form 22 June 2012

DOXORUBICIN (DOX) is one of the most effective chemotherapeutic agents used to treat leukemia, lymphomas, and solid tumors, such as breast cancer or soft tissue sarcomas. A major side effect of doxorubicin therapy is dose-dependent cardiotoxicity. Doxorubicin’s effects on cardiac energy metabolism are emerging as key elements mediating its toxicity. We evaluated the effect of doxorubicin on [14C]creatine uptake in rat neonatal cardiac myocytes and HL-1 murine cardiac cells expressing the human creatine transporter protein. A significant and irreversible decrease in creatine transport was detected after an incubation with 50–100 nmol/l doxorubicin. These concentrations are well below peak plasma levels (5 μmol/l) and within the ranges (25–250 nmol/l) for steady-state plasma concentrations reported after the administration of 15–90 mg/m2 doxorubicin for chemotherapy. The decrease in creatine transport was not solely because of increased cell death due to doxorubicin’s cytotoxic effects. Kinetic analysis showed that doxorubicin decreased Vmax, Km, and creatine transporter protein content. Cell surface biotinylation experiments confirmed that the amount of creatine transporter protein present at the cell surface was reduced. Cardiomyocytes rely on uptake by a dedicated creatine transporter to meet their intracellular creatine needs. Our findings show that the cardiomyocellular transport capacity for creatine is substantially decreased by doxorubicin administration and suggest that this effect may be an important early event in the pathogenesis of doxorubicin-mediated cardiotoxicity.

Cardiotoxicity; phosphocreatine; cardiac energy metabolism

DOXORUBICIN (DOX) is one of the most effective chemotherapeutic agents used to treat leukemia, lymphomas, and solid tumors, such as breast cancer or soft tissue sarcomas. A major side effect of the drug is dose-dependent cardiotoxicity, which may lead to chronic cardiomyopathy and congestive heart failure. DOX’s antitumor activity is thought to be primarily due to DNA damage by intercalation, breakage, alkylation, cross-linking, and inhibition of topoisomerase II (20, 30). Although all the mechanisms responsible for DOX’s cardio-toxic effects are not yet completely understood, alterations in energy metabolism appear to be important intermediaries (36). Crucial components of cardiac energy metabolism, such as AMP-activated protein kinase signaling pathways, fatty acid metabolism, mitochondrial oxidation, and creatine (Cr) kinase (CK) function are perturbed (36). Cr and its phosphorylated form phosphocreatine (PCr), together with CKs, comprise a system that helps maintain ATP stores in cardiac and skeletal myocytes (39). DOX has been shown to decrease PCr and ATP levels in isolated cardiomyocytes, perfused rat heart preparations, and living mice (19, 23, 29). These effects of DOX are similar to the pathophysiological changes generally described in patients with heart failure. In these patients, during the early stages of the disease, cardiomyocellular Cr and PCr content decrease by as much as 70%, whereas a slow and progressive ATP loss of up to 30–40% is observed during the latter stages of the disease (10, 22, 35). Thus, as heart failure progresses, the PCr-to-ATP ratio decreases (10, 12). Cr transport capacity is reduced in the failing myocardium, likely adding to the imbalance in metabolic energy that is known to be indicative of heart failure (22, 31).

The myocardium’s main source of Cr is provided by transmembrane uptake via a specific Cr transporter (CrT), which belongs to the SLC6 family of membrane proteins (21). Given the reported effects of DOX on cardiac energy metabolism, we hypothesized that DOX might inhibit CrT transport. To test this hypothesis, we studied the effects of DOX on CrT function in rat neonatal cardiomyocytes (RNCMs) and HL-1 cells using a combination of [14C]Cr uptake assays and specific immune detection of heterologously expressed human CrT protein. RNCMs comprise a primary cardiac cell culture and thus possess native cellular signaling cascades. RNCMs preferentially use glucose and lactate as energy sources over fatty acids, the main energy substrate of adult cardiomyocytes (17). The HL-1 cell line is a well-established immortalized murine atrial cell line that retains the essential hallmarks of primary cardiomyocytes and is a proven expression system to study protein structure, function, and modulation of cardiomyocytes (37). HL-1 cells have very low native Cr transport and thus are ideally suited to study Cr transport after transfection with a mammalian expression vector encoding human CrT. We (7, 8) have previously used RNCMs and HL-1 cells to characterize CrT function regulation by substrate availability and activation of AMP-activated protein kinase and PKC.

MATERIALS AND METHODS

Cell culture. RNCMs were isolated from 1- to 2-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and cultured as previously described (3). Cells were plated on tissue culture dishes precoated with gelatin-fibronectin and allowed to grow in medium 199 (Invitrogen, Carlsbad, CA) containing 2% serum before experimentation. All animals were treated according to protocols approved by the Institutional Animal Care and Use Committee of Duke University. HL-1 cells (passages 50–75) were plated onto fibronectin-gelatin-coated plates and cultured in Claycomb media (Sigma, St. Louis, MO) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM norepinephrine, and 2
mmol/L 1-g-glutamine, as previously described (37). Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

**Plasmid constructs.** Human CrT-cDNA was obtained from Dr. Marc Caron (Department of Cell Biology, Duke University). An EcoRI site followed by a Kozak sequence was added to the 5'-end, and an HindIII site was introduced at the 3'-end and subcloned into the pCDNA3.1(-) mammalian expression vector (Invitrogen). In a separate construct, an XbaI site was introduced at the 3'-end, removing the natural stop codon. This modified cDNA was then subcloned in the mammalian expression pcDNA 3.1B vector (Invitrogen). Transfection of this plasmid resulted in the expression of CrT protein bearing a COOH-terminal Myc/His tag that did not alter CrT function and was used to detect CrT-Myc protein by Western blot analysis. As a negative control for expression, cDNA encoding CrT was subcloned and was used to detect CrT-Myc protein by Western blot analysis.

The rat/human chimeric construct was prepared in two steps using PCRs. The first step consisted of two reactions. In the first reaction, the rat NH₂-terminal sequence was amplified using primers that resulted in a 200-bp fragment, including an EcoRI site, a Kozak sequence, and the 5'-end of the cDNA for rat CrT (forward primer: 5'-GAATTCCACCATGGCCAAGAAGCGCC-3', 5'-end, the forward primer corresponded to the human cDNA (reverse primer: 5'-CATGATGAAGTCCTCGCTGGTCCA-3'). The template for these reactions was a synthetic DNA fragment encoding the rat CrT NH₂-terminus. A second reaction, using human CrT cDNA as the template, generated a 700-bp DNA fragment that was complementary at the 5'-end (forward primer: 5'-TGGACGCGCCAGATGGACTTCATCATG-3') to the 3'-end of the 200-bp fragment described above and encompassed a unique Psfl site at position 1104 of the human cDNA (reverse primer: 5'-CCCTGACTCTTGCCACCTTGGGA-3').

Products of these two reactions were used as templates in the second step; the forward primer corresponded to the 5'-end of the rat CrT NH₂-terminus, and the reverse primer was the same as used in the second reaction of the first amplification round. The resulting 1,120-bp fragment was subcloned into the pSCB vector (Invitrogen) and sequenced to verify the integrity of the construct. The EcoRI to Psfl fragment was isolated and swapped with the corresponding fragment from the full-length human cDNA, creating the final chimeric plasmid. The integrity of the construct was again verified by overlapping sequencing.

**CrT protein expression in HL-1 cells.** HL-1 cells have very low native Cr transport and thus are well suited for studying Cr transport after transfection with a mammalian expression vector encoding human CrT (7). This cell line is a well-established immortalized murine atrial cell line that retains the essential hallmarks of primary cardiomyocytes and is a proven expression system to study protein structure, function, and modulation (21, 37).

Transient transfection of HL-1 cardiomyocytes was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cultures intended for Cr transport measurements were grown in 24-well culture plates, whereas those used to characterize CrT protein were cultured in 6-well dishes. Cells were allowed to grow for ~16 h after transfection before they were incubated with DOX.

**DOX incubation and [14C]Cr transport in RNCMs and HL-1 cells.** DOX at a concentration of 100 mmol/l represents the average steady-state concentration of the drug that is typically measured in patient plasma. For dose-response experiments, cultures were incubated in media containing 25, 50, 75, and 100 mmol/l DOX (Sigma) for 48 h.

Time course experiments were conducted with cells incubated for 4, 12, 24, 36, and 48 h in media containing 100 mmol/l DOX. To determine the recovery of Cr transport after DOX exposure, cells were first incubated in the presence of 100 mmol/l DOX for 4 h. The culture medium containing DOX was then removed by aspiration, and cells were washed with control media. Cr transport assays were performed after growth in control media for 0, 4, 12, 24, or 48 h. CrT protein expression were cultured in 24-well plates coated with fibronectin and gelatin. Wells were washed twice with room temperature choline buffer (150 mmol/l choline chloride, 1 mmol/l CaCl₂, 5 mmol/l MgCl₂, 2 mmol/l KCl, and 5 mmol/l HEPES-Tris; pH 7.5). Cells were then incubated for 10 min in a CO₂ incubator at 37°C in sodium uptake buffer (150 mmol/l NaCl, 1 mmol/l CaCl₂, 5 mmol/l MgCl₂, 2 mmol/l KCl, and 5 mmol/l HEPES-Tris; pH 7.5) supplemented with 0.275 μCi/ml [14C]Cr (55 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO) and unlabeled Cr to a final concentration of 15 μmol/l for dose-response and time course experiments, which is within the reported physiological range (26). Uptake was terminated by aspiration of the radiolabeled solution followed by three washes with ice-cold choline buffer. Cells were lysed with 500 mmol/l NaOH and heated to 80°C for 30 min. Cell lysates (100 μl) were subjected to scintillation counting using a Beckman Coulter LS 6500 scintillation counter. Cr transport was normalized to the protein concentration measured using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). For experiments with RNCMs, each condition was tested in quadruplicate. Measurements were performed in triplicate for experiments performed using HL-1 cells. The number of independent experiments used for data analysis is shown in each corresponding figure.

**Cr transport kinetic analysis.** For assays that measured Cr transport kinetics, the transport assay described above was performed using uptake buffers with a final Cr concentration that ranged from 5 to 305 μmol/l. Data are reported as means ± SE. Michaelis-Menten plots were generated using curve-fitting software (SigmaPlot, version 9.0, San Jose, CA).

**DOX competition assays.** For experiments designed to determine if DOX competes with Cr during the transport cycle, transport assays measuring uptake buffers with a final Cr concentration ranging from 5 to 305 μmol/l and supplemented with 2 μmol/l DOX or 2 μmol/l DOX and 100 μmol/l β-guandopropionic acid [a structural analog of Cr and an inhibitor of Cr transport with an IC₅₀ of 50 μmol/l (5)] were performed. Cells were further processed as described above. The cardiomyocytes used in these experiments had not been incubated with DOX before the transport assay.

**Immunoblot analysis.** HL-1 cells expressing CrT protein were lysed in an ice-cold solution of 150 mmol/l NaCl, 1% Triton X-100, and 50 mmol/l Tris-HCl (pH 7.4) supplemented with protease (Complete Mini protease inhibitors, Roche, Indianapolis, IN) and phosphatase inhibitors (5 mmol/l NaF, 1 mmol/l PMSF, 2.5 mmol/l Na₂P₂O₇, 50 mmol/l β-glycerol, and 1 mmol/l Na₂VO₃, Sigma). Insoluble material was separated by centrifugation at 100,000 g for 30 min at 4°C. Solubilized protein (25 μg protein/lane) was mixed with Laemmli buffer containing 10% β-mercaptoethanol, separated by SDS-PAGE on a 7% gel for experiments regarding CrT, and then transferred to nitrocellulose membranes. Blots were incubated with 150 mmol/l NaCl, 0.05% Tween 20, 10 mmol/l Tris-HCl (pH 7.4), and 5% (wt/vol) low-fat milk powder for at least 1 h at room temperature. To detect CrT protein, blots were probed with a monoclonal rat anti-human CrT antibody we developed using a genetic immunization approach (Genovac, Freiburg, Germany). The antigen for the preparation of the antibodies consisted of the first 60 amino acids of human CrT. A number of positive hybridoma clones were isolated. For these experiments, two distinct clones (4B9 and 8A6), which only detected the human isoform of CrT, were used. For CrT-Myc protein detection, blots were probed with anti-Myc antibody at 1:5,000 dilution (catalogue no. 2276, Cell Signaling Technology, Danvers, MA). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody at 1:10,000 dilution. The TATA box-binding protein band was used as a loading control and was detected using a mouse monoclonal antibody (ABCAM, Cambridge, MA). HRP-conjugated goat anti-rabbit antibody diluted 1:7,500 (catalogue no. NA 935V, GE Healthcare, Piscataway, NJ) was used as a secondary antibody to detect CrT protein bands. The secondary antibody for the
Cultures were grown in 75-cm² dishes. After extracts were prepared using a modification of the method published by Wiseman et al. (38). Cultures were grown in normal culture media.

Cell surface labeling. HL-1 cultures were grown in 75-cm² plates and transiently transfected as described above with human CrT cDNA. Cell surface proteins were biotinylated as described by Daniels and Amara (6) using the cell surface protein isolation kit from Pierce (Rockford, IL). Briefly, cell cultures were washed twice and then resuspended in ice-cold PBS. Cells were lysed with RIPA buffer (150 mmol/l NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, and 10 mmol/l Tris-HCl; pH 7.4) containing protease inhibitors for 25 min. Samples were centrifuged at 4°C for 15 min at 15,000 g. Biotinylated proteins were isolated by incubation of the supernatant with EZ-Link Immobilized Neutravidin beads (Thermo Scientific, Rockford, IL) for 60 min at room temperature. After an extensive wash, the neutravidin beads were resuspended in Laemmli sample buffer containing 100 mmol/l DTT and eluted at room temperature for 1 h. Intracellular (nonbiotinylated) CrT protein was isolated from the flowthrough of the Neutravidin beads by immunoprecipitation using a specific anti-CrT antibody. Samples were subjected to SDS-PAGE followed by Western blot analysis, as described above. The cell surface CrT protein fraction (biotinylated CrT) was then analyzed by Western blot analysis using anti-CrT antibody as described above.

Quantification of Cr and high-energy phosphates. Tissue culture extracts were prepared using a modification of the method published by Wiseman et al. (38). Cultures were grown in 75-cm² dishes. After an incubation in control or experimental media, the cell monolayer was washed twice, scrapped in ice-cold PBS, and centrifuged for 5 min at 500 g. Cell pellets were flash frozen in liquid nitrogen and stored at −80°C until extract preparation. Five hundred microliters of 2 N perchloric acid-5 mM EDTA solution were added directly to the tube containing the cell pellets. The solution froze on contact with the pellet and was allowed to thaw to ice-water temperature. The pellet was homogenized, and the slurry was vortexed vigorously followed by centrifugation at 4°C at 20,000 g for 5 min. The supernatant was neutralized with an equal volume of 2 N KOH, 150 mM TES, and 3 M KCl. The potassium perchlorate precipitate was removed by centrifugation at 4°C at 20,000 g for 2 min. The supernatant was collected, transferred to new tubes, and stored at −80°C. AMP, ADP, ATP, and PCr were measured using the modified approach described by Goutet et al. (11). In brief, samples were diluted with mobile phase A (10 mmol/l ammonium bicarbonate buffer adjusted to pH 9.4 with ammonium hydroxide in 20% acetonitrile in HPLC-grade water), filtered through a 3,000-molecular weight cutoff device, and directly injected into a liquid chromatography (LC)-electrospray ionization (ES)/multistage mass spectrometry (MS/MS) system. Chromatographic separation was accomplished on a Shimadzu 20A series HPLC (LC) equipped with ZIC-pHILIC (5 μm, 150 × 4.6) column (SeQuant). Mobile phase B was acetonitrile. The flow rate was 0.8 ml/min. ESI-MS/MS detection was performed on an Applied Biosystems/SCIEX API 4000 QTrap instrument. The following mass-to-charge ratio MS/MS transitions were followed: 346/150.8 (AMP), 426/158.8 (ADP), 506/158.8 (ATP), and 511/158.8 (PCr). [135]NaATP (TRC, Toronto, ON, Canada) was used as an internal standard for ADP, ATP, and PCr. Calibration samples containing each analyte in the range of 0.2–50 μmol/l were run before and after each batch of study samples. Linear relationship signal (analyte/internalstandard) versus nominal concentration was found for all the analytes and used for quantification. Cr was measured by a modified creatinine assay (13) on the same equipment as for the phosphorylated metabolites described above. Briefly, samples were diluted with mobile phase A (10 mmol/l ammonium formate in 0.1% formic acid; pH 2.6) and filtered through a 3,000-molecular weight cutoff device, and 10 μl were directly injected into the LC-ESI-MS/MS system. Chromatographic separation was accomplished on an Agilent ZORBAX Eclipse Plus C18, 50 × 4.6-mm, 1.8-μm column with the following eluents. The mobile phase B was acetonitrile; the flow rate was 1 ml/min. The following mass-to-charge ratio MS/MS transitions were followed: 114/44 (Cr) and 117/47 [15H2]Cr (Cambridge Isotope Laboratories). Calibration samples containing Cr in the range of 0.2–500 μmol/l were run before and after each batch of study samples. The linear relationship signal (analyte/internalstandard) versus nominal concentration was found and used for quantification. Under the acidic conditions of the assay, PCr undergoes complete hydrolysis to Cr. Thus, measured Cr values were corrected by subtracting the measure PCR levels under basic conditions.

Cytotoxicity and apoptosis assays. RNCMs or HL-1 cells transfected with CrT were incubated with 50 or 100 nmol/l DOX for up to 48 h. Toxicity was assessed by quantifying the amount of lactate dehydrogenase (LDH) present in the cell culture media using a CytoTox96 Nonradioactive Assay (Promega, Madison, WI). Maximum cytotoxicity was determined by measuring the amount of LDH released in cells incubated with the kit’s lysis buffer. The percentage of LDH released after DOX treatment was compared with that of controls.

For apoptosis detection, HL-1 and RNCM cultures were incubated with 100 nmol/l DOX for up to 48 h as described above. Apoptosis was measured using the Caspase-Glo 3/7 Assay System (Promega). Briefly, attached cells in a single 24-well plate were washed three times with ice-cold PBS and resuspended in 500 μl of 1% Triton X-100, 10% glycerol, 2 mmol/l EDTA, 137 mmol/l NaCl, and 20 mmol/l Tris (pH 8.0). A mixture of 25 μl lysis and 25 μl caspase-3/7 assay reagent was prepared, and subsequent luminescent readings were taken every 15 min for 4 h. Peak signal readings were normalized to protein content and plotted.

Statistical analyses. Data are reported as means ± SE. Michaelis-Menten plots were generated using curve-fitting software (SigmaPlot, version 9.0). Data were analyzed using nonlinear least-squares fitting and ANOVA followed by a Fisher’s test for pairwise, intergroup comparisons (STASTISTICA, version 6.0, Tulsa, OK). Correlation was assessed using the Pearson test. P values of <0.05 were considered significant.

RESULTS

DOX reduced Cr transport in RNCMs. The effects of DOX on Cr transport capacity were quantified by measuring [14C]Cr uptake in primary RNCMs. Cr transport decreased significantly within 2–4 h of exposure to 100 nmol/l DOX (Fig. 1A). To determine if the effect of DOX on Cr transport remained after DOX was removed from the culture media, Cr transport was measured at increasing time intervals after an initial exposure of 4 h to 100 nmol/l DOX. Cr transport activity did not recover even after 48 h of growth in media devoid of DOX (Fig. 1B).

DOX effects on Cr transport in HL-1 cells were dose and time dependent. HL-1 cells were used to extend our investigations of the effects of DOX on Cr transport. As mentioned previously, this cell line is a well-established immortalized murine atrial cell line with very low native Cr transport that retains the essential hallmarks of primary cardiomyocytes and is a proven expression system to study protein structure, function, and modulation (21, 37). Cr transport measured in HL-1 cells expressing human CrT protein followed Michaelis-Menten kinetics with a Vmax of 42.9 ± 5.24 mmol/mg protein and Km of 63.5 ± 2.37 μmol/l. These values are equivalent to those reported for Cr transport in other mammalian expression systems (7, 21).

The dose dependence of DOX’s effect on Cr transport was measured in HL-1 cells after an incubation for 24 h in media...
containing 25, 50, 75, or 100 nmol/l DOX. The decrease in Cr transport induced by the incubation with DOX was dose dependent. A significant decrease in Cr transport was observed in cells incubated for 24 h in media containing DOX concentrations as low as 50 nmol/l (Fig. 2A). The time dependence of DOX’s effects on Cr transport in HL-1 cells expressing CrT protein was examined by incubating cultures in 100 nmol/l DOX for 12, 24, 36, and 48 h (Fig. 2B). Cr transport decreased by 34.2% ($P < 0.05$) after 24 h of exposure and continued to fall through 48 h of observation, when transport was then maximally decreased by 53.5% ($P < 0.05$) relative to controls.

Changes in Cr transport were not due to cell death. To determine the contribution of DOX-induced cellular injury to the reduction in Cr transport observed in RNCM or HL-1 cultures incubated with 100 nmol/l DOX for up to 48 h (Fig. 2A).

Cytotoxicity was quantified using LDH release assays, and 24–36 h elapsed before any changes in LDH release were detected in RNCMs incubated in 100 nmol/l DOX. A small increase in LDH release (7% compared with control, $P < 0.05$) was detected in RNCMs after 36 h of exposure to 100 nmol/l DOX (Fig. 3B), 32 h after Cr uptake had decreased by 37% (Fig. 1A). In HL-1 cells, 24 h of incubation with the highest dose of DOX studied (100 nmol/l; Fig. 3B) increased LDH release by ~10% compared with controls. This dose of DOX decreased Cr uptake by 34–42% (Fig. 2, A and B) compared with the 31% reduction measured in these cells when they were exposed to 50 nmol/l DOX (Fig. 2A), a dose not associated with increased cell death in HL-1 or RNCM cultures (Fig. 3B, inset).

DOX reduced the $V_{\text{max}}$ and $K_m$ for Cr transport and amount of CrT protein at the cell surface. Kinetic analysis of Cr transport measured in HL-1 cells after 12 or 24 h of incubation with 100 nmol/l DOX demonstrated a significant reduction in
structural analog of Cr that inhibits Cr transport with an IC50 of 7.5 μmol/l H9262. In H9252 cells that DOX does not compete with Cr for transport by CrT. 

Figure 2. Time course and dose-response effects of DOX on HL-1 cells. HL-1 cells expressing the human Cr transporter (CrT) protein were incubated in media containing increasing concentrations of DOX for 24 h (A) or in 100 nmol/l DOX for 12, 24, 36, and 48 h (B). Cr transport was measured as described in MATERIALS AND METHODS. Data correspond to four separate experiments, each done in triplicate. *Statistically significant decrease in transport compared with controls (P < 0.05 by ANOVA and Fisher’s LSD test).

Vmax. A significant reduction in Km was also observed after 24 h of incubation with DOX (Fig. 4 and Table 1). To address the possibility that the reduction in Cr transport was due to DOX competing for transport with Cr, we determined Vmax and Km after [14C]Cr uptake assays in the presence of 2 μmol/l DOX or 2 μmol/l DOX and 100 μmol/l β-guandopropionic acid, a structural analog of Cr that inhibits Cr transport with an IC50 of 50 μmol/l (5). Acute exposure to DOX did not significantly alter Cr transport Vmax or Km (Fig. 5 and Table 2), demonstrating that DOX does not compete with Cr for transport by CrT.

Changes in Vmax can indicate changes in the cell surface population of membrane transporter proteins (27, 40). We thus determined the amount of CrT in the cell membrane using cell surface biotinylation followed by avidin affinity binding. This approach has successfully been used to identify the cell membrane-dwelling population of CrT protein (7, 15). CrT protein was detected by Western blot analysis using human-specific rat monoclonal antibodies that we generated using genetic immunization technology. Several distinct antibody clones detected the NH2-terminus of the human isoform of CrT, with a 55-kDa band corresponding to the monomer and cell surface CrT protein. The heavier CrT bands likely represent adducts (multimers) of the transporter protein. No CrT-specific bands were detected in cells transfected with a negative control or in the cells that expressed a chimeric rat-human CrT protein where the NH2-terminus was identical to that of the rat isoform (Fig. 6).

The amount of CrT at the cell surface was decreased in HL-1 cell cultures grown in media with 100 nmol/l DOX (Fig. 7, A and B). These changes in CrT protein mirrored the decrease in Cr transport capacity observed after exposure to DOX; there was a positive correlation (r = 0.69, P < 0.05, n = 12) between Vmax (Table 2) and cell surface CrT protein abundance.

DOX effects on intracellular Cr and high-energy phosphate levels. The effects of DOX on the intracellular content of Cr, PCr, ATP, ADP, and AMP were quantified by LC-MS/MS analysis performed on extracts prepared from HL-1 cultures incubated in the presence of 100 nmol/l DOX for 24 h and compared with data obtained from control cultures. The only significant difference measured was a decrease in AMP in cultures exposed to DOX (Table 3).

DISCUSSION

The mechanisms responsible for DOX-associated acute and chronic cardiotoxicity are not clearly understood. Acute toxicity has been attributed, at least in part, to apoptosis caused by oxidation-induced mitochondrial damage and alterations in iron homeostasis (14, 32, 36). The development of chronic cardiomyopathy is presumed to be the result of the combined effects of multiple processes, such as abnormalities in Ca2+ handling, peroxidation of membrane lipids, and alteration of cardiac-specific gene expression (20, 30).

Deficits in energy metabolism have emerged as significant contributors to DOX-related cardiomyopathy. It was recently reported that DOX’s adverse effects on cardiac energy metabolism antedate the development of functional and morphological abnormalities associated with DOX’s cardiotoxicity (18). DOX treatment decreases myocardial high-energy phosphates in animal models and causes the ratio of PCr to ATP to fall in humans (19, 36). Cardiac mitochondria appear particularly susceptible to the deleterious effects of DOX. DOX reduces both the function and expression of mitochondrial enzymes (1, 2), resulting in a metabolic pathway shift characterized by a decrease in the utilization of fatty acid oxidation (β-oxidation) in favor of less-efficient glucose oxidation and glycolysis for ATP production (1, 2, 4). This switch in cardiac energy metabolism may represent an adaptative response of the cardiomyocyte at the early stages of DOX cardiotoxicity (33). The resulting ATP deficit would be compounded further by a disruption of the functional coupling between mitochondrial production of ATP and regeneration and recycling of PCr and ADP caused by a DOX-induced decrease in adenine nucleotide translocator function (24). In addition, the cardiac isoform of mitochondrial CK (MiCK) appears to have an increased sensitivity to DOX (34). DOX inhibits the binding of MiCK to cardiolipin in mitochondrial membranes and thus promotes the dissociation of subunits constituting the functional octameric assembly of MiCK (28). Impaired Cr transport may further
compromise energy production pathways in the cardiomyocyte exposed to DOX: not only will reduced Cr stores hamper the cell’s ability to maintain an adequate PCr pool and thus to buffer ATP, but it will also diminish the stimulatory effect Cr has on respiration by coupling PCr and ATP production to ADP recycling (32).

In this study, we demonstrate that DOX reduced Cr transport capacity in RNCMs and in HL-1 cardiomyocytes expressing human CrT protein. Kinetic analyses and competition assays (Tables 1 and 2) showed that DOX did not compete with Cr for transport and that in HL-1 cells, the decrease in Cr transport is the result of a reduction in transport $V_{\text{max}}$ and changes in the affinity for Cr. The decreases in $V_{\text{max}}$ observed in cardiomyocytes incubated in the presence of DOX were mirrored by decreased CrT protein at the cell surface. These findings suggest that DOX may interfere with normal trafficking and/or the stability of CrT at the cell membrane. The molecular mechanism responsible for DOX’s effect on CrT could be a result of oxidative damage due to carbonylation or nitrosylation of sensitive amino acids in the transporter protein or a yet to be identified interacting and/or modulatory protein.

The decrease in Cr transport measured did not translate to major alterations in intracellular Cr or high-energy phosphate metabolites (Table 3). These results are consistent with the change in intracellular Cr content expected based on the normal rate of Cr loss, 2% of the total cellular Cr content (16, 25), and alterations in $V_{\text{max}}$ and $K_m$ of Cr transport recorded during 24 h of incubation with DOX. In this scenario, the predicted decrease in the content of intracellular Cr would be ~1%. This is in line with our observations (Table 3) and earlier reports demonstrating that there is a lag of 24/48 h before decreases in PCr and ATP are measured in cultured cardiomyocytes exposed to DOX (9, 29).

Although the technique (LC-MS/MS) used for detecting high-energy phosphates is
compared with controls. One-way ANOVA indicated that both were incubated for 12 or 24 h with media containing 100 nmol/l DOX and HL-1 cells incubated with 100 nmol/l DOX for 12 h (condition. Each data point represents the mean ± SE of three culture wells/experimental condition.

very sensitive, it is possible that very small changes in these metabolites may not have been detected.

In RNCMs, Cr transport reduction was observed within 4 h of incubation in media containing 100 nmol/l of the chemotherapeutic agent. In HL-1 cells expressing human CrT, although statistically significant reductions were detected within 24 h of incubation in 100 nmol/l DOX, the observed decrease in Cr transport was not simply the result of increased cytotoxicity or apoptosis. For example, Cr transport capacity was substantially depressed in RNCMs incubated with 100 nmol/l DOX for 4 h, a time point where neither LDH release nor apoptosis were increased.

Similar findings were noted in HL-1 cells, where the increase in LDH release was disproportionately less than the observed reduction in Cr transport after the incubation with DOX (Figs. 2B and 3B). It is also important to note that transport was significantly reduced after incubation with a DOX concentration (50 nmol/l) that did not cause detectable cytotoxic effects. Finally, correlation analysis revealed no correlation (in HL-1 cells) or a negative correlation (in RNCMs) between either the decrease in $V_{\text{max}}$ of Cr transport and LDH release or caspase-3/7 activation, indicating that the reduction in Cr transport capacity was not closely associated with cell death or apoptosis.

The adverse effects of DOX on Cr transport in RNCMs were evident even after the cells were no longer in the presence of the drug; recovery of transport to control levels was not observed even after 48 h of incubation in control media. This persistence in alterations after removal of DOX has been reported for several other proteins and transcripts isolated from rat hearts exposed to DOX (2) and has been attributed to impaired transcription of specific genes together with oxidative damage to the proteins themselves. This may well be the case in RNCMs, which express the native CrT protein. ROS derived from DOX’s metabolic processing in the mitochondria may affect not only the CrT protein itself (and/or proteins necessary for its trafficking or Table 1. Effect of DOX on Cr transport $V_{\text{max}}$ and $K_m$

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<tr>
<th>Condition</th>
<th>Relative $K_m$</th>
<th>Relative $V_{\text{max}}$</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>12-h DOX</td>
<td>0.83 ± 0.09</td>
<td>0.75 ± 0.08*</td>
</tr>
<tr>
<td>24-h DOX</td>
<td>0.75 ± 0.08*</td>
<td>0.63 ± 0.08*</td>
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Values are expressed as means ± SE of 3 independent experiments each done in triplicate. Doxorubicin (DOX) altered creatine (Cr) transport kinetics in HL-1 cells expressing human Cr transporter (CrT) protein. Kinetic parameters were calculated as described in MATERIALS AND METHODS. HL-1 cultures were incubated for 12 or 24 h with media containing 100 nmol/l DOX and compared with controls. One-way ANOVA indicated that both $K_m$ and $V_{\text{max}}$ were affected by DOX. *Significant difference compared with controls ($^*P < 0.05$ by ANOVA and Fisher’s least-significant-difference test).

Table 2. DOX does not compete with Cr for transport by CrT

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<tr>
<th>Condition</th>
<th>Relative $V_{\text{max}}$</th>
<th>Relative $K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>DOX</td>
<td>1.09 ± 0.36</td>
<td>1.76 ± 0.69</td>
</tr>
<tr>
<td>β-GPA</td>
<td>0.43 ± 0.11</td>
<td>25.01 ± 3.04*</td>
</tr>
<tr>
<td>β-GPA + DOX</td>
<td>0.47 ± 0.23</td>
<td>22.40 ± 5.95*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE of 3 independent experiments each done in triplicate. Kinetic analysis demonstrated that in HL-1 cultures, DOX (2 µmol/l) in either the presence or absence of β-guanidopropionic acid (β-GPA) does not significantly change $V_{\text{max}}$ or $K_m$ of Cr transport compared with the control. As expected, 100 µmol/l of the structural analog β-GPA decreased Cr transport, as shown by the significant increase in $K_m$. *Significant difference compared with the control and DOX ($P < 0.05$ by ANOVA and Fisher’s least-significant-difference test).
stability to the cell surface) but also its transcription, resulting in a pervasive decrease in Cr transport capacity.

The DOX concentrations that were used in this study are 40–100 times lower than the peak concentrations (5 μmol/l) typically measured in the serum of patients receiving DOX infusions of 60–90 mg/m2 and are within the recorded range for steady-state plasma concentrations (25–250 nmol/l) reported for those individuals. Circulating concentrations of DOX fall from their initial peak values to ~50 nmol/l within 72 h of drug administration (12). The time frame that was used to study DOX’s effects on Cr transport was also well within the half-life reported for circulating DOX. *Significant difference compared with the control (P < 0.05 by Student’s t-test).

In conclusion, this is the first report of a deleterious effect of exposure to comparatively low concentrations of DOX on Cr transport in cardiomyocytes. A marked decrease in Cr transport occurs after initial drug administration and at concentrations that are similar to, or lower than, those experienced by patients during a standard course of chemotherapy. These findings appear to be primarily the result of an irreversible decrease in the availability of CrT on the cell surface. The elucidation of the molecular mechanisms responsible for this reduction in Cr transport warrants further investigation. Cr transport may represent a target for early therapeutic intervention to preserve Cr and PCr levels in the myocardium, which could mitigate DOX’s cardiotoxicity in patients.

ACKNOWLEDGMENTS

This effort is dedicated to Helen M. Jacobs, who died of doxorubicin-induced heart failure.

The authors gratefully acknowledge Dr. Raul E. Cachau for insights and expertise at the early stages of this study, Dr. Dawn E. Bowles for helpful comments and discussion of the manuscript, and Dr. Ivan Spasojevic and Karel Basel from the Pharmacokinetics/Pharmacodynamics Bioanalytical Core Laboratory (Duke Cancer Institute) for assistance with LCMS/MS analysis. The

<table>
<thead>
<tr>
<th>Cr, μmol/mg protein</th>
<th>PCr, μmol/mg protein</th>
<th>ATP, μmol/mg protein</th>
<th>ADP, μmol/mg protein</th>
<th>AMP, μmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.75 ± 2.47</td>
<td>6.43 ± 1.21</td>
<td>0.74 ± 0.20</td>
<td>0.60 ± 0.07</td>
</tr>
<tr>
<td>DOX</td>
<td>23.01 ± 7.92</td>
<td>5.94 ± 0.12</td>
<td>0.60 ± 0.15</td>
<td>0.50 ± 0.07</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE of 3 independent experiments each done in triplicate. The content of Cr, phosphocreatine (PCr), ATP, ADP, and AMP was measured by liquid chromatography-tandem mass spectroscopy, as described in MATERIALS AND METHODS. Equal amounts (25 μg) of protein were loaded in each lane. TATA-binding protein (TBP) was used as a loading control.
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authors also acknowledge Quique Toloza for assistance in the preparation of the manuscript.

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REFERENCES

This work was funded by the Department of Surgery of Duke University Medical Center.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


