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ORIGINAL ARTICLE

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Prevention of chronic anthracycline cardiotoxicity in the adult Fischer 344 rat by dexrazoxane and effects on iron metabolism

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Abstract *Purpose*: Anthracyclines, such as doxorubicin and daunorubicin, continue to be widely used in the treatment of cancer, although they share the adverse effect of chronic, cumulative dose-related cardiotoxicity. The only approved treatment in prevention of anthracycline cardiotoxicity is dexrazoxane, a putative iron chelator. Previous in vitro studies have shown that disorders of iron metabolism, including altered IRP1–IRE binding, may be an important mechanism of anthracycline cardiotoxicity. *Methods*: This study examined the role of IRP1–IRE binding ex vivo in a chronic model of daunorubicin cardiotoxicity in the Fischer 344 rat and whether dexrazoxane could prevent any daunorubicin-induced changes in IRP1 binding. Young adult (5–6 months) Fischer 344 rats received daunorubicin (2.5 mg/kg iv once per week for 6 weeks) with and without pretreatment with dexrazoxane (50 mg/kg ip). Other groups received saline (controls) or dexrazoxane alone. Rats were killed either 4 h or 2 weeks after the last dose of daunorubicin to assess IRP1–IRE binding. *Results*: Contractility (dF/dt) of atrial tissue, obtained from rats 2 weeks after the last dose of daunorubicin, was significantly reduced in daunorubicin-treated compared to control rats. Dexrazoxane pretreatment protected against the daunorubicin-induced decrease in atrial dF/dt. However, left ventricular IRP1/IRE binding was not affected by daunorubicin treatment either 4 h or 2 weeks after the last dose of daunorubicin. *Conclusions*: IRP1 binding may not be altered in the rat model of chronic anthracycline cardiotoxicity.

Keywords Anthracyclines · Daunorubicin · Cardiotoxicity · Rat · Iron regulatory protein · Dexrazoxane

The authors state there are no conflicts of interest regarding the work in this paper.

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Introduction

The mechanism of anthracycline-mediated cardiotoxicity remains controversial [30, 31]. It is now apparent that anthracycline-mediated alterations in iron metabolism may contribute to the cardiotoxicity [41]. This idea is supported by several important studies. Dexrazoxane, a putative iron chelator, protects against chronic anthracycline cardiotoxicity, in both animals and humans [17, 18, 36, 37]. Likewise, cardioprotection is afforded by desferroxamine, another iron chelator [19, 38]. Other in vitro studies have demonstrated that anthracyclines disturb the normal regulation of iron metabolism in the cell. Normal cellular iron flux is tightly regulated by the interaction of iron regulatory proteins (IRP1 and IRP2) with untranslated *cis*-sequences of iron-responsive elements (IRE) of ferritin and transferrin receptor1 (TrF1) mRNA [16, 32]. Increased binding of IRP to these IREs (in response to low cellular iron) stabilizes the otherwise unstable TfR1 mRNA and inhibits ferritin translation, with a net result of increasing TrF1 and decreasing ferritin,

thereby augmenting the cellular labile iron pool. When cellular iron is replete, the bifunctional IRP1 is inactivated by reassembling the 4Fe–4S cluster in the protein so that IRP1 loses the ability to bind to IRE and acquires aconitase function. IRP2 activity is lost by proteosomal degradation in iron-replete cells. The resultant loss of IRP/IRE binding lowers TrF1 and raises ferritin, producing a decrease in the labile iron pool.

Awareness of the potential effects of anthracyclines on iron metabolism was raised by Minotti et al. [27, 28] who reported that doxorubicinol has the ability to delocalize Fe^{2+} from the aconitase 4Fe–4S cluster in human heart cytosol preparations and regenerate the parent anthracycline, while releasing low molecular weight Fe^{2+}, which can become available for further oxidation reactions. Further study of the effect of doxorubicin and doxorubicinol on human myocardial cytosolic samples demonstrated that not only did doxorubicinol abolish aconitase activity, but that doxorubicin irreversibly inactivated IRP1 by an effect of doxorubicin–Fe(II) complexes to form a ''null protein'' incapable of IRE RNA binding or of reconstituting aconitase activity [28]. Additional generation of reactive oxygen species by the anthracycline [29], together with the non-physiological increase in cytoplasmic iron from IRP1 activation, sets the stage for cytotoxicity, including apoptosis, as has been demonstrated in bovine endothelial cells [22]. The toxic effect of anthracycline on endothelial cells was abrogated by transferrin receptor antibody, indicating the importance of transferrin receptor function and iron transport in the cytotoxicity [22].

Subsequent investigations in intact cell systems have demonstrated a more complicated picture, however. Using neonatal rat cardiomyocytes incubated with doxorubicin, Kwok and Richardson [23] noted a concentration-dependent reduction in active IRP1/IRE binding following 6 h of incubation; the effect was not seen after 24 h incubation, however. The observed loss of IRP1 activity was explained by the formation of doxorubicin–iron or doxorubicin–copper complexes that caused a reversible decrease in active IRP–RNA binding. In other studies using H9c2 embryonic rat heart cell culture, doxorubicin and daunorubicin produced a bell-shaped, dose-related effect on IRP1–IRE binding so that the binding was increased at lower concentrations of anthracycline and decreased towards control levels at higher concentrations of anthracycline [29]. Thus the effect of doxorubicin on IRP/IRE binding appears to vary according to conditions of treatment in vitro. Whether this effect of anthracycline is of mechanistic importance in a model of chronic cardiotoxicity is not known. The present study was designed to determine whether IRP1/IRE binding in the heart is altered in a model of chronic daunorubicin cardiotoxicity in the adult Fischer 344 rat.

Materials and methods

Animals

Healthy male Fischer 344 rats aged 5–6 months were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA). They were housed for at least 1 week to acclimatize and for monitoring of health before inclusion in the study. The rats were housed, two per cage, with an ambient temperature of 21°C. The rats were given water ad libitum and fed standard rat chow (Tekland Rodent Blox, Hubbard, OR, USA) for the period of the study. The welfare of animals was protected and the local animal care and use committee approved the study protocol.

Protocol

The rats were assigned to one of the four treatments with weekly intravenous daunorubicin (Cerubidine®) or saline with pretreatment with intraperitoneal dexrazoxane (Zinecard®) or dexrazoxane vehicle. One group received daunorubicin by weekly intravenous bolus injection with prior treatment with intraperitoneal sodium lactate (dexrazoxane vehicle) given 30 min before the daunorubicin injection. A second group received weekly daunorubicin doses as above with prior treatment with dexrazoxane ip given 30 min before the daunorubicin. A third group received weekly iv daunorubicin vehicle (0.9% saline) with prior treatment with ip dexrazoxane. A fourth group received weekly iv daunorubicin vehicle with pretreatment with dexrazoxane vehicle. Each dose of daunorubicin was administered by iv bolus administration over 1 min into a hind leg vein and dexrazoxane was administered ip 30 min prior to daunorubicin treatment. Volumes of vehicles administered alone matched the volumes of vehicle given with the corresponding drug. The dose of daunorubicin in rats was 2.5 mg/kg iv weekly for 6 weeks to reach a total of 15 mg/kg. The respective dose of dexrazoxane was 50 mg/kg in young rats, which was 20 times the dose of daunorubicin. This dose ratio previously has been shown to ameliorate chronic anthracycline cardiotoxicity in the rat [13, 14, 21].

Matched feeding was achieved by averaging the daily amount of food consumed by daunorubicin-treated rats, and this amount was then provided to other groups. Matched feeding was carefully maintained to avert possible confounding effects of nutritional differences on heart function [15, 20, 34, 35] or on anthracycline pharmacokinetics [7] between the groups. Body weights were monitored weekly.

Rats were killed either 4 h or 2 weeks after the last dose of daunorubicin by guillotine decapitation. The heart was quickly removed and a left ventricular tissue sample (approximately 100 mg) was obtained and

placed in RNA *later*® solution (Ambion, Austin, TX, USA) and instantly frozen in liquid nitrogen for IRE1/IRP studies. Left ventricular tissue samples (approximately 100 mg) were obtained from all hearts and quickly frozen in liquid nitrogen for the determination of anthracycline drug concentrations.

In young and old hearts obtained 2 weeks after the last dose of daunorubicin, left atria were excised and affixed to an isometric force transducer in a muscle bath of Krebs-bicarbonate buffer containing 127 mM NaCl, 2.5 mM $CaCl_2$, 2.3 mM KCl, 25 mM $NaHCO_3$, 1.3 mM KH_2PO_4, 0.6 mM $MgSO_4$, and 5.6 mM glucose, pH 7.4, that was maintained at 30°C and continuously bubbled with a 95% O_2, 5% CO_2 gas mixture. Cardiac functional parameters at different rates of contraction were measured as described below.

Cardiac function studies

Left atria were affixed to force transducers (Kulite BG25, Leonia, NJ, USA) and electrically stimulated to contract isometrically using square wave pulses (Grass S88, Quincy, MA, USA) at 10% above threshold voltage. Atria were stretched to the baseline resting force of 0.5 g. Preparations were stabilized at a contraction frequency of 60 per minute (1 Hz). Stabilization was judged to have occurred when there was no significant change in resting or developed force for at least 60 min. Data were recorded using a Gould 2400S physiological recorder and a Buxco (Troy, NY, USA) pulsatile analyzer. The contractile parameters measured included resting force (RF) as an index of preload and the maximum rate of change of force (dF/dt) as a measure of contractility. Contractile parameters in atria were measured at stimulation rates of 15, 60, and 120 contractions per minute (cpm). After the final measurement, the buffer was drained from the muscle bath, and the muscles were washed three times with fresh buffer. Krebs-bicarbonate buffer containing 0.5 mM Ca^{2+} was then added to the bath and contractile function was evaluated. This process was repeated, using replacement buffers with increasing Ca^{2+} concentrations (0.5, 1.5, 2.5, 3.5, and 4.5 mM). Following each buffer replacement, the atria were allowed to equilibrate at 60 cpm for 20 min stabilization periods before measuring contractile function.

Drug assay methods

Cardiac tissue samples were assayed for daunorubicin and daunorubicinol concentrations, using doxorubicin as an internal standard, based on methods previously reported [6, 8, 9]. Standard curves for daunorubicin and daunorubicinol were obtained by adding purified anthracyclines to drug-naïve rat tissue. Daunorubicinol for use in standard curves was extracted from rat kidney homogenates that had been incubated for 8 h in a bath containing 5 μg/ml daunorubicin [9].

Samples were organically extracted as described previously [8]. For analysis of drug levels in rat heart tissue, samples (stored at −70°C) were extracted following homogenization and addition of doxorubicin (1,000 ng) as internal standard. After addition of chloroform:isopropyl alcohol (5 ml, 1:1 v/v) and ammonium sulfate (3 g), anthracyclines were extracted from samples by vortexing, then shaking for 30 min, followed by centrifugation (15 min at 2,000 rpm = 830×*g*) and removal of the upper organic phase to a new tube. The extract was evaporated under a stream of nitrogen and re-dissolved in initial condition solvent [200 μl; ammonium formate buffer (pH 4):acetonitrile 76:24, v/v]. Samples (100 μl injection volume) were assayed by high-performance liquid chromatography using a Phenomenex 5 μ Luna® phenyl–hexyl column (4.5 mm×150 mm), with a solvent gradient (linear gradient to 70:30 ammonium formate buffer:acetonitrile by 8 min), and fluorescence detection. Solvent flow rate was 2 ml/minute, and doxorubicin, daunorubicinol, and daunorubicin were eluted at 6.4, 7.5, and 10.7 min, respectively. All procedures occurred in polypropylene tubes and samples were protected from light. The molar extinction coefficient of 1.15×10^4 m^{-1} cm^{-1} at 485 nm was utilized for doxorubicin, daunorubicin, and daunorubicinol standards. Standard curves were developed both for daunorubicin and daunorubicinol in naive heart tissue. Levels of anthracyclines were calculated as microgram per gram of heart tissue. Recovery of anthracyclines ranged from 20 to 30% (dependent upon anthracycline species). The HPLC assay employed a Waters automated gradient HPLC system (Waters Corp., Milford, MA, USA), a Shimadzu RF-10AXL fluorescence detector (Columbia, MD, USA) equipped with a xenon lamp, and a Hewlett Packard 3390A Integrator (Avondale, PA, USA). Signal detection was carried out at $\lambda_{ex}470/\lambda_{em}550$.

Cellular extraction

Left ventricular myocardium (approximately 50 mg) was homogenized in a polytron blender for 30–45 s in 5 ml of 50 mM Hepes, pH 7.5, 3 mM $MgCl_2$, 40 mM KCl containing 5% glycerol. After centrifugation at 12,000×*g* for 20 min at 4°C, the supernatant was again centrifuged at 110,000×*g* for 1 h. The cytosolic supernatant was aliquoted and stored at −80°C for gel mobility shift assay.

RNA–protein gel retardation assay

The 59 nucleotide [^{32}UTP]-labeled ferritin IRE RNA probe was synthesized from an EcoR1 digest of pTZ19 (MBI Fermentas, Vilnius, Lithuania) by in vitro transcription according to protocol. The probe was electrophoresed on a 6% acrylamide/bis-acrylamide (19:1) gel for 1 h at 250 V. The radiolabeled band was eluted overnight at 37° C in 400 μl of 2 M ammonium acetate,

1% SDS containing 10 μg yeast t-RNA. The probe was precipitated with 2.5 volumes of ethanol, washed in 75% ice-cold ethanol, and redissolved in TE buffer. Approximately 10,000 cpm of this transcript was incubated at room temperature with 2 μg protein containing IRP1 from each cytosolic extract in 25 mM Hepes, pH 7.6, 250 mM potassium acetate, 1.5 mM $MgCl_2$, and 5% glycerol for 30 min in a final volume of 20 μl. Heparin (75 μg per assay) was added for 15 min to exclude non-specific binding prior to separation of IRP1/IRE complexes by non-denaturing gel electrophoresis on a 6% acrylamide/bis-acrylamide (19:1) gel for 3 h at 100 V. Samples were quantitated by densitometry (Molecular Dynamics Densitometer SI, Sunnyvale, CA, USA). Parallel experiments were performed in samples pretreated with 9 mM cysteine as a reducing agent before the addition of the RNA probe to reconstitute IRP1 binding to IRE, thus assaying the total IRP1 in the sample. Three samples from all four groups were included in each gel shift assay. A reference sample with and without cysteine from one rat was included in each assay to reduce day-to-day variation. The duration of exposure without cysteine was about twice the duration of exposure for samples pretreated with cysteine, since cysteine reconstitution led to higher IRP/IRE binding activity. Preliminary assays were performed to verify the specificity of IRP/IRE binding. Inclusion in the assay of 0.1 or 1 μg of non-radiolabeled IRE RNA as a specific competitor effectively abolished IRE binding. However, no competition was observed using identical levels of yeast t-RNA as the non-specific competitor (data not shown).

Data analysis

Mean comparisons of clinical data were made by Student's *t* test or by ANOVA with Tukey's post hoc test. The null hypothesis was rejected when $P < 0.05$.

Results

The rats treated with daunorubicin lost a significant amount of weight (Fig. 1) from 339 ± 7 g at the beginning of the study to 262 ± 7 g at the time of killing ($P < 0.001$). Weight loss in the other pair-fed young groups was also significant, but less than in the daunorubicin-treated group ($P < 0.05$). Thus, the weight loss in the daunorubicin-treated rats reflected the toxicity from daunorubicin in addition to decreased food intake. This additional weight loss due to daunorubicin was prevented by pretreatment with dexrazoxane.

There was a significant effect of daunorubicin treatment on atrial contractility (dF/dt) in atria contracting at 15 cpm (Fig. 2). Two weeks following completion of chronic daunorubicin treatment (15 mg/kg over 6 weeks) dF/dt in atrial preparations was significantly decreased compared with the control group (10.1 ± 1.0 vs. 32.9 ± 3.1 g/s; $P < 0.001$; Fig. 2a). Pretreatment with dexrazoxane prevented this decline in contractility due to daunorubicin, with similar values for dF/dt at 15 cpm (26.9 ± 2.9 g/s) compared to the control group (32.9 ± 3.1 g/s). The dexrazoxane-treated group was also similar to controls. It is highly unlikely that the difference in dF/dt between treatment groups at 15 cpm is explained by a difference in resting force since (a) despite the statistical difference in resting force (Fig. 2b), there was no physiologically significant difference between groups and (b) dexrazoxane pretreatment prevented the decline in dF/dt without any change in RF compared with the daunorubicin-treated group (Fig. 2b). Thus, chronic treatment with daunorubicin caused impairment of atrial contractility that was prevented by dexrazoxane pretreatment.

Chronic daunorubicin treatment also blunted the inverse relationship between the rate of contraction and contractility. Thus the increase in dF/dt that occurred

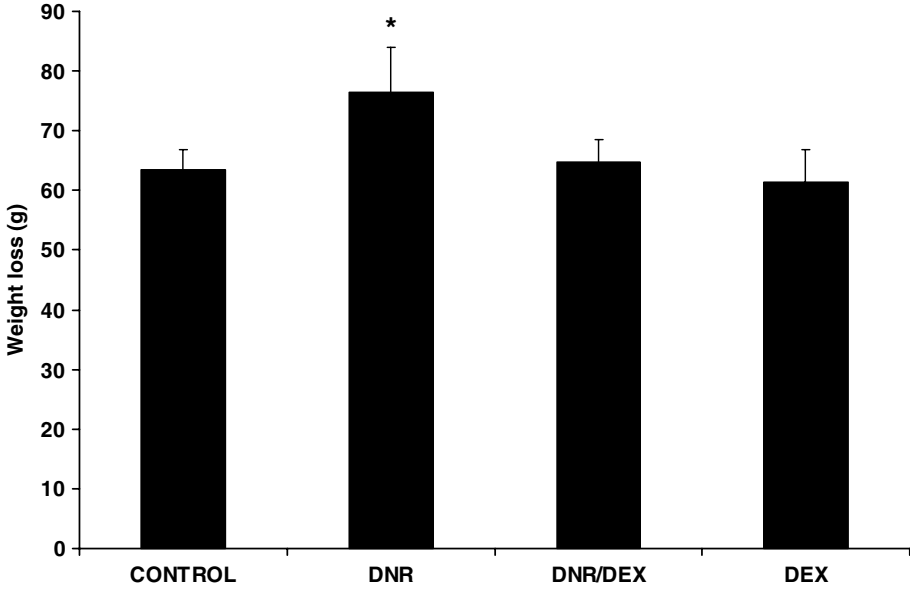

Fig. 1 Weight loss in Fischer 344 rats at the end of the 8-week study. Groups were vehicle (*Control*), daunorubicin (*DNR*), daunorubicin + dexrazoxane (*DNR/DEX*), and dexrazoxane (*DEX*). Drugs were administered over the first 6 weeks. All groups were pair-fed to match food intake in the daunorubicin-treated group. *$P < 0.05$ vs. control. Values are mean ± SEM

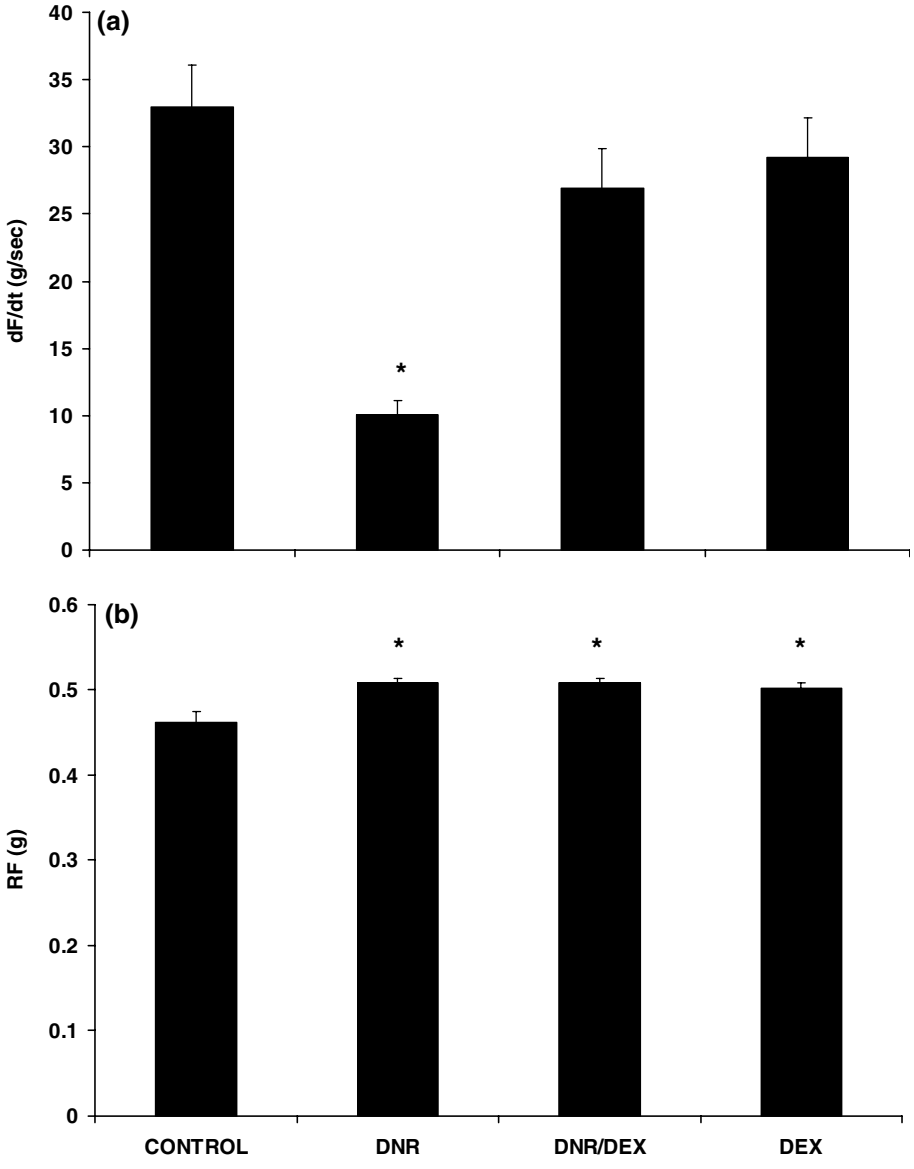

Fig. 2 Effect of chronic daunorubicin treatment on **a** contractility (dF/dt) and **b** resting force (RF) in atrial strips from Fischer 344 rats obtained 2 weeks after the last dose of treatment in control (*Control*), daunorubicin-treated (*DNR*), daunorubicin + dexrazoxane-treated (*DNR/DEX*) and dexrazoxane-treated (*DEX*) rats. $*P < 0.001$ vs. control. Values are mean ± SEM. (*Control* = 8, *DNR* = 11)

with a decline in the rate of contraction (120, 60, and 15 cpm) was reduced by daunorubicin administration (Fig. 3). With each decrement in the rate of contraction from 120 to 15 cpm, contractility rose significantly in control atria ($P < 0.05$). However, daunorubicin treatment blunted the effect of rate, with a significant difference between the dF/dt at 15 cpm in the daunorubicin-treated and control rats (40 ± 9 vs. $113 \pm 18\%$, respectively; $P < 0.05$). There was no significant difference between the other treatment groups compared with controls. Thus, chronic daunorubicin treatment blunted the inverse effect of rate on contractility (dF/dt), and this effect of daunorubicin was prevented by dexrazoxane pretreatment.

We also studied the effect of incremental concentrations of Ca^{2+} on atrial contractility (Fig. 4). Contractility (dF/dt) rose with the increase in Ca^{2+} from 0.5 to 4.5 mM in the incubation buffer. In atria from control rats, the increase in dF/dt over the Ca^{2+} range (0.5–4.5 mM) went from 10.5 ± 2 to 30.0 ± 1.8 g/s ($P < 0.001$). The inotropic effect of Ca^{2+} in the daunorubicin-treated rats (3.8 ± 0.4 to 12.6 ± 1.5 g/s) was significantly less than in controls ($P < 0.05$). Dexrazoxane pretreatment prevented this inhibitory effect of daunorubicin (Fig. 4).

The effect of daunorubicin treatment on IRP activity in Fischer 344 rats is shown in Fig. 5. The IRP activity was measured in heart samples obtained both 4 h and 2 weeks after the last dose of daunorubicin or equivalent treatment in other groups. Representative gels showing IRP1–IRE binding in left ventricular tissue from three animals in each treatment group 4 h after the completion of chronic treatment with daunorubicin is shown in the upper panel. Active binding in the upper line (a) and total binding in reconstituted IRP1 samples after cysteine treatment in the lower line (b) did not differ between

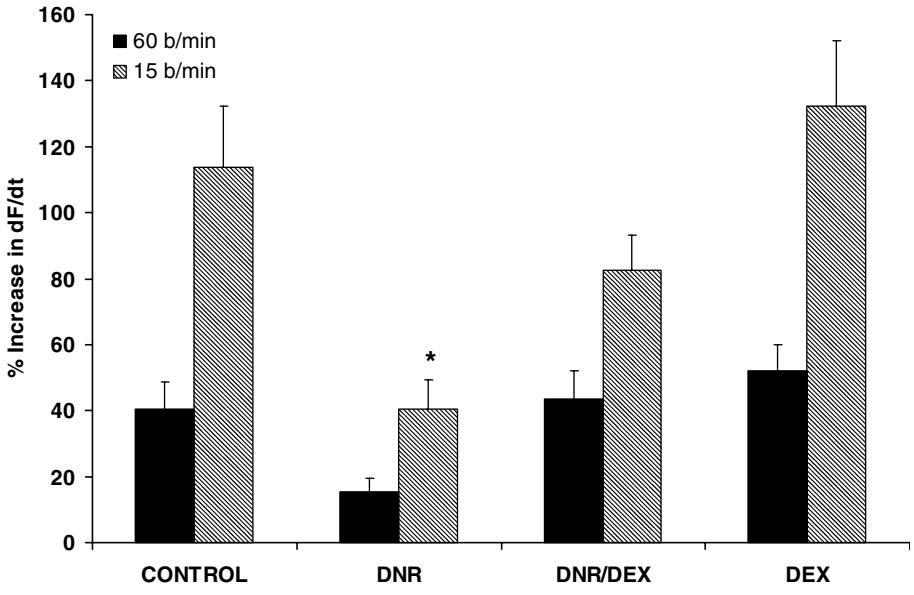

Fig. 3 Effect of rate of contraction on contractility (dF/dt) in atrial preparations from adult Fischer 344 rats treated for 6 weeks with daunorubicin vehicle (*Control* = 8), daunorubicin (*DNR* = 11), daunorubicin + dexrazoxane (*DNR/DEX* = 9), and dexrazoxane (*DEX* = 9) over 6 weeks. *Bars* show the percentage increase in dF/dt at 60 cpm (1 Hz) and 15 cpm (0.25 Hz) in comparison with 120 cpm (2 Hz). $*P < 0.05$ vs. control. Values are mean ± SEM

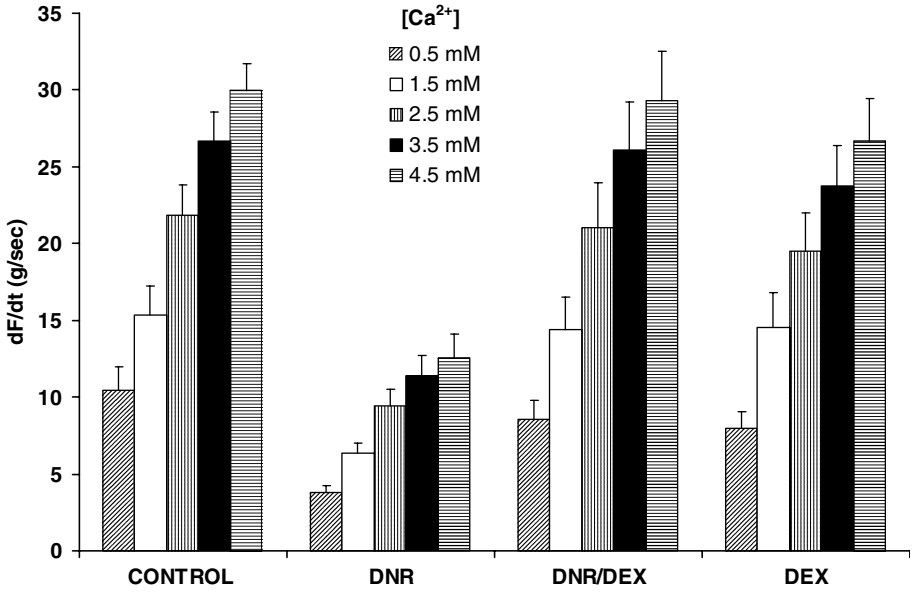

Fig. 4 Effect of buffer Ca^{2+} concentration (0.5–4.5 mM) on dF/dt in atrial preparations from Fischer 344 rats treated for 6 weeks with daunorubicin vehicle (*Control* = 8), daunorubicin (*DNR* = 11), daunorubicin + dexrazoxane (*DNR/DEX* = 9), and dexrazoxane (*DEX* = 9) over 6 weeks. Atria were stimulated to contract at 15 cpm (0.25 Hz). The dF/dt increased significantly with Ca^{2+} concentration ($P < 0.001$). The inotropic effect of Ca^{2+} was significantly reduced by daunorubicin treatment compared to control ($P < 0.05$), but not by other treatments. Values are mean ± SEM

controls and daunorubicin-treated animals. The assay at 4 h after the dose was performed to evaluate the acute effect of drug and metabolite on IPR activity when cardiac concentrations of both daunorubicin and daunorubicinol are near their highest levels [9, 10], to potentially maximize the effect on IRP1–IRE binding [30]. However, at 4 h there was no effect of daunorubicin on quantitative IRP activity compared to control (Fig. 5, middle panel). Dexrazoxane treatment was associated with a decrease in IRP activity ($P < 0.05$). In the presence of cysteine used to reconstitute IRP activity, there was a small, but insignificant increase in activity in the daunorubicin-treated group. The IRP1 activity was similar to the control in other groups. Samples obtained 2 weeks after the dose were assayed to detect any residual effect of chronic treatment on IRP activity at a time when drug concentrations are not detectable or are at very low levels (Table 1). Activity of IRP observed 2 weeks after the last treatment did not differ between treatment groups and control either in the absence or presence of cysteine (Fig. 5, lower panel). Since dietary intake was reduced in all groups, with resultant decreased intake of minerals including iron, we were concerned that this reduction in iron intake might have upregulated IRP1–IRE binding activity in all groups and thereby distorted the possible effect of daunorubicin treatment [3, 4]. However, there was no difference between IRP1–IRE binding in pair-fed rats and rats fed a normal diet at the end of the 6-week treatment period (data not shown).

Both daunorubicin and daunorubicinol concentrations were assayed in ventricle samples obtained 4 h and

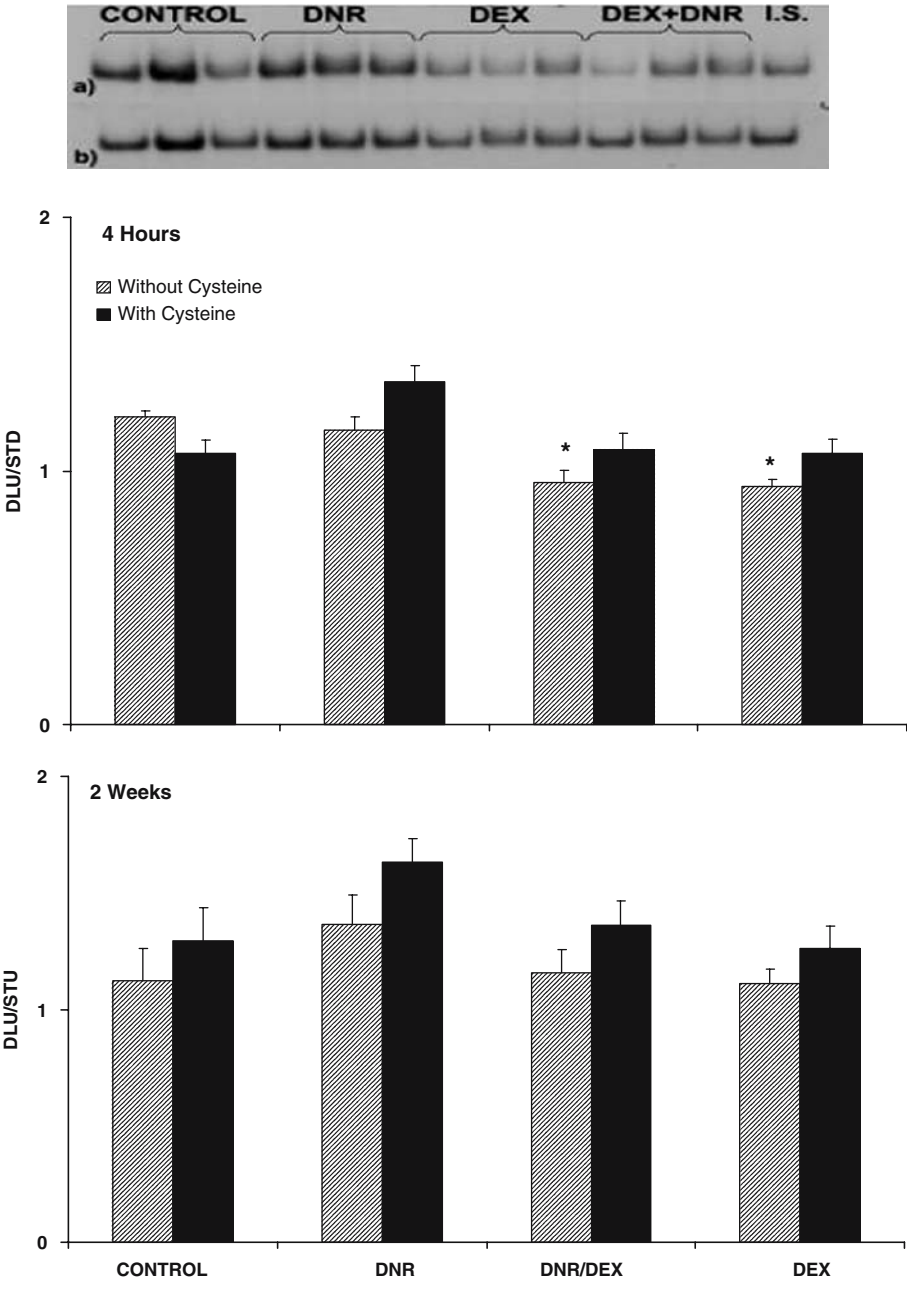

Fig. 5 A representative gel shift assay at 4 h is shown in *upper panel* with *a* active binding without cysteine in the *upper line* and *b* total binding with cysteine (a reducing agent that restores full IRE binding) in the *lower line*. Note that the density for total is not increased relative to active binding because of reduced exposure time. The gels show control and treatment groups with $n = 3$ in all groups performed together on the one gel along with an internal standard (*I.S.*) that was used as a reference to account for any day-to-day variation in the assay. Iron regulatory protein-1/iron response element binding activity assessed by gel retardation assay in ventricular tissue obtained from Fischer 344 rats 4 h (*middle panel*) and 2 weeks (*lower panel*) following chronic treatment with daunorubicin vehicle (*Control*; 4 h = 10; 2 week = 8), daunorubicin (*DNR*; 4 h = 10; 2 weeks = 11), daunorubicin + dexrazoxane (*DNR*/*DEX*; 4 h = 10; 2 weeks = 11), and dexrazoxane (*DEX*; 4 h = 10; 2 weeks = 9) over 6 weeks. Binding is shown as active (*hatched bars*) without cysteine and as total (*solid bars*) with cysteine. $*P < 0.05$ vs. control. Values are mean ± SEM. Data for binding are expressed as a ratio of a control sample (+/− cysteine) that was measured each day of the gel shift assay

Table 1 Concentrations of daunorubicin and daunorubicinol in heart tissue 4 h and 2 weeks after the last dose of chronic daunorubicin treatment in Fischer 344 rats with and without pretreatment with dexrazoxane

Group	Time after dose	Daunorubicin (μg/g tissue)	*n*	Daunorubicinol (μg/g tissue)	*n*
DNR	4 h	1.82 ± 0.08	10	1.99 ± 0.10	10
DNR + Dex	4 h	1.78 ± 0.25	10	2.23 ± 0.29	10
DNR	2 weeks	0.06 ± 0.01	6	0.04 ± 0.01	6
DNR + Dex	2 weeks	0.09 ± 0.03	5	0.04 ± 0.01	6

DNR daunorubicin, *Dex* dexrazoxane

2 weeks after the last dose of daunorubicin, with or without pretreatment with dexrazoxane (Table 1). Consistent with prior pharmacokinetics studies [12], there was no effect of dexrazoxane on the cardiac concentrations of daunorubicin or daunorubicinol.

Discussion

While many studies have addressed the changes in IRP/IRE binding in vitro in cell and cell-free conditions

[5, 22, 23, 28, 29], this is the first study to determine IRP/IRE binding in an in vivo model of chronic anthracycline cardiotoxicity. In this study, no effect of chronic anthracycline treatment on cardiac IRP/IRE binding was observed, either 4 h or 2 weeks after the last dose of daunorubicin (Fig. 5). This suggests, contrary to in vitro observations [5, 22, 23, 27–29], that chronic anthracycline treatment sufficient to cause contractile dysfunction (Figs. 2, 3, 4) is not associated with altered IRP1 activity in the heart. These apparently contradictory findings may not be mutually exclusive, however. This apparent discrepancy between our study and the in vitro studies may result from differences in condition of exposure to anthracycline, differences in concentrations of anthracycline or to cell or tissue specificity in IRP1–IRE binding effects due to anthracyclines. For example, irreversible inactivation of IRP has been demonstrated in human myocardial cytosolic fractions exposed to doxorubicinol 4 nmol/mg protein [28]. The action of doxorubicinol on aconitase and IRP1 required the presence of *cis*-aconitate as an intermediate substrate of aconitase in the reaction buffer. The effect of doxorubicinol was dependent on the concentration of *cis*-aconitate, being maximal at 100 μM *cis*-aconitate. These in vitro conditions may not occur in the rat model of chronic daunorubicin cardiotoxicity.

In addition, in H9c2 cells (embryonic, rat heart derived cell line), the effect of daunorubicin incubation on IRP1/IRE binding was related to the concentration of anthracycline in the incubation medium, with an actual increase in IRP1/IRE activity in the presence of daunorubicin up to 15 μM, then declining with further increases in concentration [29]. A similar bell-shaped pattern was seen for doxorubicin. Thus anthracyclines both increase and decrease IRP1 activity depending on the concentration. In neonatal cardiomyocytes used to study the effect of anthracyclines on IRP/IRE binding, Kwok and Richardson [23] observed that doxorubicin had little effect on binding in media concentrations of 1–10 μM but caused a decrease in binding at 20 μM concentration. The effect was also time dependent; IRP1 binding was reduced at 6 h but not at 24 h. They also observed cell specificity in anthracycline effect on IRP1 binding; more significant effects of doxorubicin were detected in SK-Mel-28 melanoma cells than in cardiac myocytes. Human tumor cell lines also demonstrate cell type specificity in the effect of doxorubicin on IRP1 binding [1]. Thus, effects seen in vitro in cell-free systems, in H9c2 cells, or neonatal cardiomyocytes may not necessarily be observed in the heart of the intact rat because of these factors.

To investigate the potential confounding effect of timing of assay of IRP1 binding, we analyzed IRP1 binding 4 h after the last dose of daunorubicin. This time of dose was selected to coincide with the highest cardiac concentrations of daunorubicinol seen after intravenous bolus injection of daunorubicin [9, 10]. This time also closely approximated the time of anthracycline exposure that caused the largest effect in neonatal cardiomyocytes [23] and in bovine aortic endothelial cells [22].

Were daunorubicin and daunorubicinol concentrations in the heart in vivo high enough to produce an effect on IRP1 binding? The anthracycline concentrations in the medium of cell preparations associated with alterations in IRP1/IRE binding were in the range of 0.5–20 μM [22, 23, 29]. Average cardiac concentrations of daunorubicin and daunorubicinol in heart obtained 4 h after the last dose in our study were approximately 3.3 and 3.6 μM and 2 weeks after the last dose were 0.1 and 0.07 μM, respectively (Table 1). How these concentrations relate to intracellular concentrations achieved in isolated cell preparations in medium incubated with 0.5–20 μM anthracycline is not known. They may be considerably less [22, 23, 29], since cardiomyocytes in vitro can accumulate intracellular daunorubicin to levels 30–40 fold higher than the buffer concentration [2]. Thus anthracycline concentrations in the intact chronic heart model may not be sufficient to elicit an effect on IRP1 binding.

It is also possible that with chronic anthracycline treatment, counter regulation of IRP1 activity by factors such as dietary intake of iron may have occurred to offset the direct effect of anthracycline. The effect of iron deficiency on intracellular IRP1 activity [3, 4] is unlikely to have occurred in our experiments since additional experiments showed no difference in IRP1 binding at the end of the 6-week treatment period between pair-fed rats (that consumed a similar dietary intake to that given to the animals in our experiment) and rats fed a normal diet (data not shown).

While IRP1 has been the focus of intracellular iron regulation due to its relative abundance, regulation activities, and binding affinities, recent reports suggest that IRP2 may be the predominant regulator of intracellular iron. There is evidence that IRP2 knockout mice develop progressive brain degeneration with misregulation of expression of target proteins in all tissues while IRP1 knockouts do not demonstrate overt pathology. Instead the effects of IRP1 deficiency are localized, with misregulated expression of targets in kidney and brown fat, two tissues where expression of IRP1 greatly exceeds that of IRP2 [25]. Cellular iron response to hypoxia and iron deficiency is marshaled via IRP2 rather than IRP1, indicating that IRP2 may be the more important adaptive iron sensor [24, 25]. Thus, IRP2, which is also affected by anthracycline treatment [22, 29], may be the more important target for intracellular regulation of iron in chronic anthracycline cardiotoxicity.

We observed a decline in IRP1–IRE binding in rat heart 4 h after the last dose of daunorubicin in both dexrazoxane-treated groups compared to control, but not at 2 weeks after the last dose of daunorubicin (Fig. 5). This observation of a decrease in IRP1–IRE binding with dexrazoxane treatment is surprising since it was anticipated that the iron binding properties of dexrazoxane would cause low cellular iron and a secondary elevation in IRP–IRE binding as demonstrated

in leukemic cell lines by Weiss et al. [39]. Likewise, Kwok and Richardson [23] showed that desferroxamine produced an increase in IRP binding in melanoma cells, but the effect of upregulation of IRP–IRE binding was not seen at 6 h and did not occur until incubation with desferroxamine for 24 h. Incubation with dexrazoxane in leukemia cells can cause upregulation of transferrin receptor and increased iron influx into the cell [39]. Increased iron influx could in turn produce a reduction in IRP1–IRE binding. Whether such a response is "primed" in animals treated chronically with dexrazoxane is not known, since this model of effect of dexrazoxane has not been evaluated previously.

There was impairment of the negative staircase effect of contraction rate on contractility in the daunorubicin-treated animals (Fig. 3), similar to previous observations [11]. The findings of altered rate-related inotropic effect are suggestive of altered sarcoplasmic reticulum Ca^{2+} metabolism, since similar rate-related effects were prevented by ryanodine [26]. Likewise, the inotropic effect of Ca^{2+} was diminished by daunorubicin treatment (Fig. 4). This may also relate to sarcoplasmic reticulum Ca^{2+} handling since the increase in buffer Ca^{2+} enhances Ca^{2+} transients [33], thereby augmenting Ca^{2+}-induced Ca^{2+} release by the sarcoplasmic reticulum [40]. Dexrazoxane, by ameliorating the daunorubicin-induced impairment of rate and $[Ca^{2+}]_o$-induced inotropy, appears to prevent the sarcoplasmic reticulum dysfunction in a chronic model of anthracycline cardiotoxicity.

In summary, this study demonstrates for the first time that, contrary to previous in vitro studies, chronic anthracycline cardiotoxicity in the rat does not appear to be associated with changes in IRP1–IRE binding activity at either 4 h or 2 weeks after the last dose of daunorubicin. Dexrazoxane prevents the decline in atrial contractility due to chronic daunorubicin treatment.

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