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Research Article

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Doxorubicin Cardiomyopathy Is Associated with a Decrease in Calcium Release Channel of the Sarcoplasmic Reticulum in a Chronic Rabbit Model

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Abstract

Doxorubicin is a highly effective cancer chemotherapeutic agent that produces a dose-dependent cardiomyopathy that limits its clinical usefulness. Clinical and animal studies of morphological changes during the early stages of doxorubicin-induced cardiomyopathy have suggested that the sarcoplasmic reticulum, the intracellular membrane system responsible for myoplasmic calcium regulation in adult mammalian heart, may be the early target of doxorubicin. To detect changes in the calcium pump protein or the calcium release channel (ryanodine receptor) of the sarcoplasmic reticulum during chronic doxorubicin treatment, rabbits were treated with intravenous doxorubicin (1 mg/kg) twice weekly for 12 to 18 doses. Pair-fed controls received intravenous normal saline. The severity of cardiomyopathy was scored by light and electron microscopy of left ventricular papillary muscles. Developed tension was measured in isolated atrial strips. In subcellular fractions from heart, [^{3}H]ryanodine binding was decreased in doxorubicin-treated rabbits (0.33±0.03 pmol/mg) compared with control rabbits (0.66±0.02 pmol/mg; $P < 0.0001$). The magnitude of the decrease in [^{3}H]ryanodine binding correlated with both the severity of the cardiomyopathy graded by pathology score (light and electron microscopy) and the decrease in developed tension in isolated atrial strips. B_{max} for [^{3}H]ryanodine binding and the amount of immunoreactive ryanodine receptor by Western blot analysis using sequence-specific antibody were both decreased, consistent with a decrease in the amount of calcium release channel of sarcoplasmic reticulum in doxorubicin-treated rabbits. In contrast, there was no decrease in the amount or the activity of the calcium pump protein of the sarcoplasmic reticulum in doxorubicin-treated rabbits. Doxorubicin treatment did not decrease [^{3}H]ryanodine binding or the amount of immunoreactive calcium release channel of sarcoplasmic reticulum in skeletal muscle. Since the sarcoplasmic reticulum regulates muscle contraction by the cyclic uptake and release of a large internal calcium pool, altered function of the calcium release channel could lead to the abnormalities of contraction and relaxation observed in the doxorubicin cardiomyopathy. (*J. Clin. Invest.* 1993. 91:1697–1705.) **Key words: binding • cardiac • chemotherapy • radioligand • ryanodine receptor**

Introduction

Doxorubicin is a highly effective cancer chemotherapeutic agent, but its clinical usefulness is limited due to the development of a dose-dependent cardiomyopathy (1–7). The total dose is usually limited to 450–500 mg/m^2 body surface area, since the incidence of the cardiomyopathy is "low" below this dose. However, more than half of the patients could tolerate higher total doses (potentially needed to treat their malignancies) without development of cardiomyopathy, whereas a small percent of patients will develop the cardiomyopathy at even these low doses (8, 9). The abnormalities of contraction and relaxation associated with this cardiomyopathy are unique. For example, some patients that develop congestive heart failure during doxorubicin chemotherapy exhibit a slow improvement in cardiac function, which suggests that the doxorubicin-induced cardiac dysfunction may be reversible (10–12). In contrast, a small percentage of patients with normal cardiac function after completing doxorubicin chemotherapy develop myocardial dysfunction years later (13, 14). Prevention of this cardiomyopathy will require a more detailed characterization of its etiology.

Endomyocardial biopsy has been used to monitor for the doxorubicin cardiomyopathy. Billingham (15) described the morphological changes seen on biopsy specimens from patients receiving doxorubicin. The earliest changes are distended sarcoplasmic reticulum and early myofibrillar loss (15, 16). Later changes suggest diffuse cell damage with degeneration of multiple cellular organelles. The morphological changes on biopsy appear to precede overt heart failure (15, 17). These early morphologic abnormalities of the sarcoplasmic reticulum have been described in animal models as well (18–23).

The sarcoplasmic reticulum regulates the intracellular calcium stores on which adult mammalian cardiac muscle is dependent for contraction. The calcium pump protein, which is involved in energized calcium uptake enabling muscle to relax, and the calcium release channel, which mediates calcium release to trigger muscle contraction, are the two key proteins involved (24). Thus, a number of studies have focused on the in vitro effects of anthracyclines on function of the pumps and channels of this subcellular membrane system. Doxorubicin induces calcium release from isolated sarcoplasmic reticulum vesicles and in skinned cardiac fibers (25–30). [^{14}C]-Doxorubicin binds to the calcium release channel in fractions enriched in terminal cisternae (31, 32). Doxorubicin also increases open probability of calcium release channels in reconstituted lipid bilayers (33–35). Doxorubicinol, a metabolite of

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doxorubicin, is a potent inhibitor of multiple intracellular pumps, including the calcium pump protein of the cardiac sarcoplasmic reticulum in isolated sarcoplasmic reticulum vesicles (36). However, higher anthracycline concentrations appear to be required to mediate effects on the calcium-dependent ATPase than on the calcium release channel (28, 36). Thus, there is compelling evidence that anthracyclines alter the function of the sarcoplasmic reticulum in vitro, suggesting that the contractile dysfunction of anthracycline-induced cardiomyopathy might be mediated by similar effects in vivo.

Recent studies have also suggested that acute in vitro and prolonged in vitro exposure have different effects. Single channel studies have demonstrated that although short exposures to doxorubicin open the channel, prolonged exposures irreversibly close the channel (35). In isolated rat ventricle it was also demonstrated that acute in vitro exposure enhances [^{3}H]-ryanodine binding and calcium release in a reversible manner whereas prolonged (5–24 h) in vitro exposure increases doxorubicin sensitivity of the calcium release channel in an irreversible manner (28). However, the time course of these "prolonged" exposures is still relatively short in comparison to the time course of exposure to the drug during chemotherapy. The relevance of these in vitro observations to the etiology of the chronic doxorubicin cardiomyopathy seen clinically remains to be established.

The purpose of this study was to assess the effects of chronic doxorubicin administration on sarcoplasmic reticulum function and to correlate changes in sarcoplasmic reticulum function with functional and microscopic evidence of cardiotoxicity.

Methods

Rabbits develop a dose-dependent cardiomyopathy with morphologic changes similar to those described in humans (21, 37–41). Adult New Zealand White rabbits (1.9–3.7 kg) were randomly assigned to a doxorubicin treatment group (n = 20) or a pair-fed control group (n = 20). Doxorubicin (1 mg/kg) (Adria Laboratories, Columbus, OH) was infused into an ear vein over 30 min twice weekly for a total of 12 to 18 doses. The dose was chosen as that producing clinical effect as evidenced primarily by a significant drop-off in food intake. Pair-fed control rabbits were infused with normal saline on the same schedule.

Body weight and food intake were monitored throughout the experiment. Pair feeding was carefully maintained to ensure that there was no significant contribution of malnutrition to observed differences between doxorubicin-treated and control-fed rabbits. The weight of food consumed by the treated rabbit was measured every 1–2 d and determined the amount of food given the control-fed rabbit at the next feeding. The treated rabbits were less active than their paired controls but were otherwise healthy without signs of infection. Blood cell counts were obtained on half the pairs and no significant differences in hematocrit (treated: 33±1% vs. control: 36±1%), white cell counts (treated: 8.5±0.2/mm^2 vs. control: 7.6±0.7/mm^2), or platelet counts (treated: 544±106 vs. control: 369±49) were observed. This is consistent with a lack of severe bone marrow suppression with the doxorubicin treatment protocol used.

At the conclusion of the treatment protocol (1–34 d after last dose), the rabbits were anesthetized with pentobarbital (50 mg/kg) and anticoagulated with heparin (100 U/kg). The hearts were removed through a sternotomy and immediately placed in Krebs bicarbonate buffer with the following composition (mM): 5 dextrose, 142 Na^+, 3.6 K^+, 0.6 Mg^+, 25 HCO_3^-, and 2.5 Ca^{2+} at 30°C. A left atrial strip was suspended from a jewelers chain on a force transducer (Gould, Inc., Glen Burnie, MD) with the lower end anchored against bipolar stimulus electrodes, placed in a bath with the Krebs bicarbonate buffer (as above) at 30°C bubbled with 95% O_2/5% CO_2, and stimulated at 0.5 Hz for 30 min to allow stabilization. The atria were stretched to a resting force of 0.38 g. After stabilization, tracings were recorded on a strip chart at 100 mm/s paper speed and were later digitalized and analyzed by computer. The heart was then placed in normal saline solution on ice. A left ventricular papillary muscle was removed and placed in glutaraldehyde solution for light microscopy and electron micrograph (EM)[1] examination. The atria were removed, frozen, and used for quantitation of doxorubicin and doxorubicinol levels. The remaining left ventricle and right ventricle were homogenized and a sarcoplasmic reticulum–enriched microsomal fraction was isolated by using the method of Chamberlain and Fleischer (42) modified in this laboratory for rabbit heart. Modifications included initial homogenization with a Polytron (30 s × 2) and the inclusion of protease inhibitors (0.2 mM PMSF and 0.5 μg/ml leupeptin) in the homogenization buffer. The microsomal fractions were stored at −70°C (early in the study) or at −120°C (later in the study) until used for the specific assays. Protein concentrations were determined using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL).

With the last several rabbit pairs, a sarcoplasmic reticulum–enriched microsomal fraction was also isolated from rabbit back muscle by the methods of Saito et al. as described in Chu et al. (43).

Light and EM scoring. Left ventricular papillary muscles were scored in a blinded fashion by a cardiovascular pathologist (J.B. Atkinson). The severity of doxorubicin-induced myopathic changes was graded by light microscopy and confirmed by EM, according to the method of Billingham (15). Overall scores for each rabbit were derived by examination of 5 to 10 blocks.

Ryanodine binding. Recent identification of ryanodine as a specific high-affinity ligand for the calcium release channel (24) provided a method to screen for changes in regulation of the open state of the calcium release channel as well as for changes in amount of calcium release channel in relatively small and impure preparations. Ryanodine binding is calcium dependent, felt to reflect that channel opening is calcium dependent and ryanodine binds only to the open channel. In vitro, doxorubicin increases ryanodine binding (and, by inference, calcium release channel opening) by increasing calcium sensitivity of the channel in subcellular fractions enriched in sarcoplasmic reticulum, as previously described (28). We used radioligand binding to quantitate and evaluate several parameters of the calcium release channel of potential functional significance in chronically treated rabbits.

Total ryanodine binding to microsomal fractions was measured after incubation with [^{3}H]ryanodine for 60 min at 37°C (44). Nonspecific binding was measured by incubation with > 100-fold excess cold ryanodine. Specific high-affinity ryanodine binding was calculated as the difference between total and nonspecific binding. Vesicle-bound [^{3}H]ryanodine was separated from free [^{3}H]ryanodine by ultracentrifugation in a TL-100 Tabletop System (Beckman Instruments, Palo Alto, CA) with a TLA-100 rotor (44a) at 245,000 g for 10 min. The binding buffer was 1M KCl, 10 mM Hepes, 25 μM CaCl (pCa 4.7) at pH 7.4, which was adjusted to various lower pCas by the addition of EGTA. Ryanodine binding for each heart (treated or control) was performed at 10 nM ryanodine concentration. The binding isotherms were performed on pooled treated or pooled control vesicle fractions. For the in vitro doxorubicin studies, doxorubicin was added to microsomal fractions before the addition of ryanodine.

Western blot with calcium release channel–specific antibody. A sequence-specific polyclonal antibody was raised in rabbit that cross-reacted with both the skeletal and cardiac calcium release channels. Cardiac (6 μg) and skeletal (4 μg) microsomal fractions or cardiac homogenate (24 μg) were electrophoresed under denaturing conditions on gradient (5–15%) minigels and were transferred overnight onto transfer membrane (Immobilon-P; Millipore Corp., Bedford,

1. *Abbreviation used in this paper:* EM, electron micrograph.

MA). After preincubation in Blotto-based blocking solution for 60 min (45), the membranes were incubated for 90 min with immune or preimmune serum in Blotto-based blocking solution followed by alkaline phosphatase-linked goat anti-rabbit antibody in Blotto-based blocking solution and were developed with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt (Sigma Chemical Co., St. Louis, MO).

Quantitation of the sarcoplasmic reticulum calcium pump and its activity. Microsomal fractions were run in duplicate or triplicate with treated and control pairs on the same gradient polyacrylamide minigel (5–15%) and were stained with Coomassie blue. The relative amounts of protein in the 100 kD band, corresponding to the calcium pump protein in treated versus control fractions, was estimated by densitometry using the MicroScan Gel Analysis Program and Densitometer by Technology Resources Inc. (Nashville, TN).

Calcium-dependent ATPase and the ATP-dependent oxalate-stimulated calcium loading of the calcium pump protein were measured as previously described (36). ATP-stimulated calcium uptake was measured by incubation in buffer (20 mM Imidazole-HCl, 100 mM KCl, 4 mM $MgCl_2$, 5 mM NaN_3, 100 mM sucrose, 30 μM EGTA, 0.1 mM ouabain, 2.5 mM K_2-oxalate, 66.2 μM Ca^{2+} or 1.0 mM EGTA, ±3.5 mM Na_2ATP) for 2 min, followed by isolation of the vesicles by filtration. The ATP-stimulated ^{45}Ca loaded was then measured in a scintillation counter. Simultaneously, calcium-stimulated ATPase activity was determined by the difference in ATPase activity in presence of Ca^{2+} (66.2 μM) and in the absence of Ca^{2+} (1.0 mM EGTA). Activity is expressed per milligram of total protein.

Quantitation of Mg^{2+}-dependent ATPase activity and Na^+K^+ ATPase activity. Mg^{2+}-dependent ATPase activity was measured in the microsomal fraction by methods previously described (46). Magnesium-dependent ATPase activity was measured by incubation in buffer (33 mM Tris-acetate, pH 7.4, and 3.5 mM Na_2ATP) for 4 min at 30°C in the presence and absence of Mg^{2+} (1.7 mM).

Ouabain-sensitive Na^+K^+ ATPase activity was measured in the microsomal fraction by methods previously described (47). ATPase activity was measured by incubation in buffer with the following composition (mM): 30 imidazole-HCl, 120 NaCl, 20 KCl, 3 $MgCl_2$, 0.5 EGTA, 5 NaN_3, and 2 Na_2ATP for 10 min at 37°C in the absence and presence of ouabain (1 mM).

Measurement of cardiac concentrations of doxorubicin and doxorubicinol. Tissue samples were blotted, weighed, and stored at −20°C until transported on dry ice for analysis of drug levels. Drug levels were measured in a blinded fashion. The tissue samples (50–100 mg) were added to 2 g ammonium sulfate and 3 ml 0.9% NaCl, homogenized (1 min) and then spiked with daunorubicin (5–100 μg as required) as an internal standard. 5 ml of isopropanol/chloroform (50:50, vol/vol) was added to the homogenization mixture, vortexed for 3 min, and centrifuged at 500 *g* for 12 min. The organic layer was extracted again using chloroform/isopropanol containing 3 M silver nitrate, vortexed, and centrifuged as above. The organic layer was separated, dried under nitrogen, and resuspended in 500 μl methanol before HPLC.

The HPLC system (Waters Associates, Milford, MA) uses a 4 μm phenyl Radial-Pak reversed-phase column (Waters Associates) with gradient control of the mobile phases (initially 72:28, vol/vol 0.1% ammonium formate buffer (pH 4.0)/100% acetonitrile; and finally 100% acetonitrile) at a flow rate of 3 ml/min. Detection is by a fluorescent detector (Kratos Analytical Instruments, Ramsey, NJ) with excitation at 470 nm and emission at 550 nm. Standard curves with varying doxorubicin or doxorubicinol concentrations were prepared using untreated rabbit ventricular tissue.

Statistical analysis. Comparisons between treated and control data were performed by unpaired *t* test, with the exception that the pathology scores were compared using rank-sum analysis (NCSS Statistics Package, Kaysville, UT). Values are expressed as mean±standard error. The doxorubicin dose–response curves and the pCa curves were generated from the data using a computer program (Graphpad, San Diego, CA) to obtain the best-fit sigmoid curve.

Results

Characterization of the degree of myopathy. Food intake fell off significantly in the treated rabbits from the first week (155 g/d) to the final week (70 g/d). By virtue of the design of the pair-feeding protocol, there was no significant difference in the weight of the control rabbits (3.31±0.08 kg) and the treated rabbits (3.06±0.10 kg) at the end of the treatment protocol. In contrast, the weight of the hearts (ventricles) was significantly higher in the treated rabbits (5.0±0.2 g) compared with the control rabbits (4.4±0.1 g) ($P < 0.02$), and the ratio of heart weight to body weight was also significantly higher for the treated rabbits (1.66±0.08) than the control rabbits (1.35±0.03) ($P < 0.002$). Heart weight and degree of myopathy are correlated (Table I).

The primary microscopic changes observed in ventricles from treated rabbits was sarcotubular swelling without mitochondrial or myofibrillar loss (Fig. 1). Comparing the pathology scores for doxorubicin cardiomyopathy, the left ventricular papillary muscles from the treated rabbits (1.31±0.14) demonstrated early changes consistent with doxorubicin cardiomyopathy that were not observed in the pair-fed controls (0.15±0.03) (highly significant by rank-sum test). To allow comparison of various parameters with degree of myopathy, the rabbits were arbitrarily subdivided into mild, moderate, and severe myopathy according to their microscopic score (Table I). A score of < 1 was considered mild, 1–2 was moderate, and > 2 was severe.

Heart (ventricles) and atrial weight in the mildly affected rabbits were not significantly different from the controls, but the moderate and severe groups demonstrated a progressive rise in these weights (Table I). Severely affected hearts were visibly enlarged at the time of removal, whereas the mild and moderate groups were similar in appearance to the controls.

Table I. Comparison of Severity of Cardiomyopathy

	EM score	Heart weight	Atria weight	Developed tension (0.5 Hz)	Developed tension (max)	[^{3}H]Ryanodine binding
		g	*g*	*g/mg*	*g/mg*	*pmol/mg*
Control	0.15±0.03 (15)	4.50±0.10 (15)	0.58±0.03 (15)	0.097±0.010 (13)	0.144±0.011 (13)	0.631±0.027 (11)
Mild	0.69±0.15 (4)	4.44±0.26 (4)	0.62±0.05 (4)	0.058±0.006‡ (4)	0.125±0.023 (4)	0.415±0.093 (3)
Moderate	1.26±0.09 (8)	5.07±0.22* (8)	0.64±0.03 (7)	0.043±0.013‡ (7)	0.135±0.023 (7)	0.313±0.063‡ (4)
Severe	2.23±0.12 (3)	5.99±0.18§ (3)	1.32±0.17‡ (3)	0.013±0.006§ (3)	0.031±0.014§ (3)	0.204±0.024§ (3)

Parentheses indicate *n* for particular measurement. * $P < 0.05$. ‡ $P < 0.01$. § $P < 0.001$.

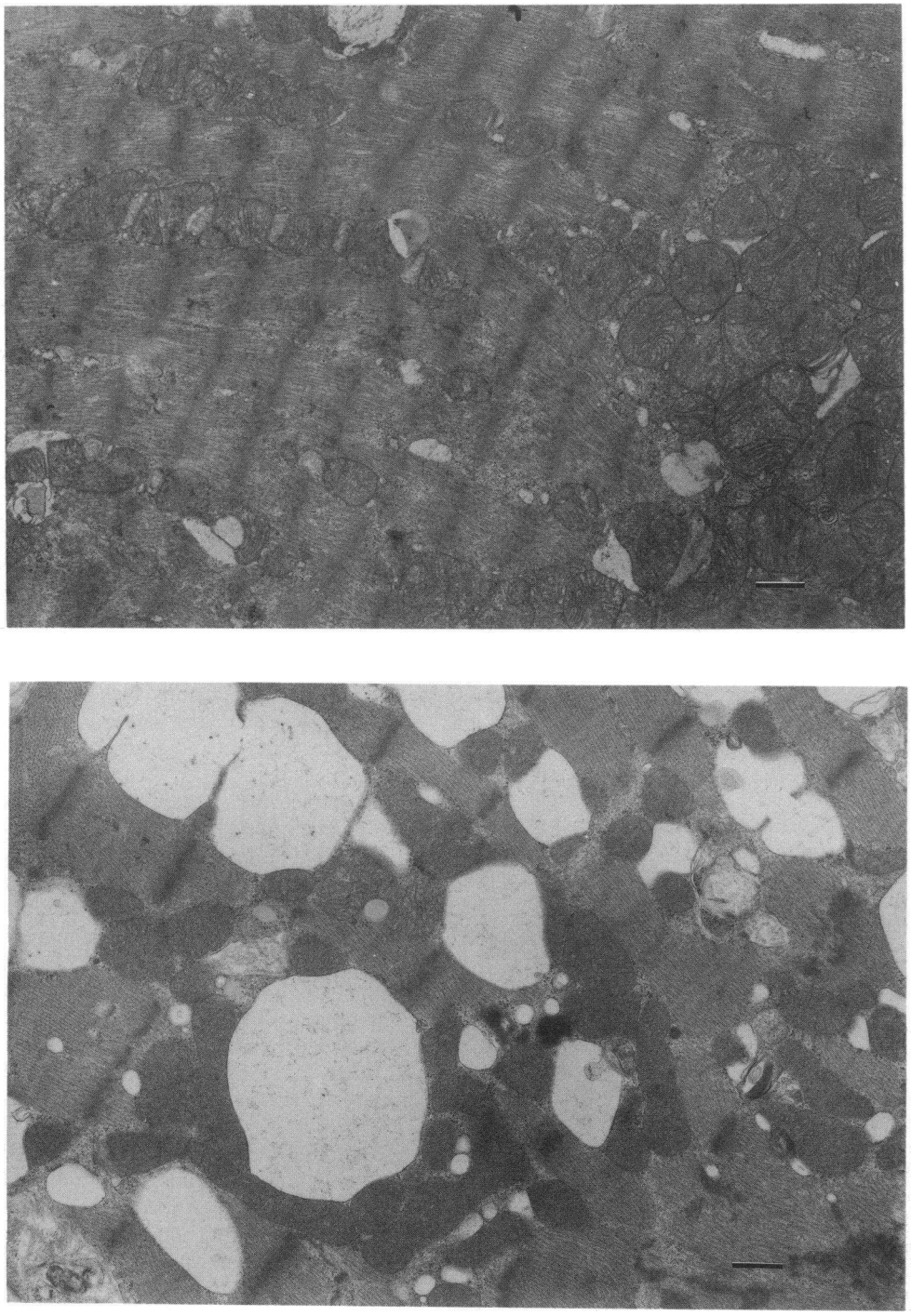

Figure 1. (*a*) EM of a control rabbit demonstrating normal myocyte structure. Bar, 1 μm. (*b*) EM of a treated rabbit demonstrating severe dilatation of the sarcotubular structures (pathology score = 1.8). This was the common finding in doxorubicin-treated rabbits, with myofibrillar loss being rare. Bar, 1 μm.

Functional changes in intact isolated muscles. Left atrial strips were of equal size in the treated (8.2±0.9 mg) and control (8.8±1.2 mg) rabbits. While stimulating at 0.5 Hz, the developed tension (g/mg atria) was decreased in the treated versus the control rabbits (Table I). Increasing severity of the myopathy by pathology score was associated with decreasing developed tension at this frequency. Thus, there was good correlation between the extent of inhibition of contractile function and severity of microscopic lesions.

Characterization of the subcellular fractions. Although the protein yield of the microsomal fraction was higher for the treated than the control rabbits, there was no significant difference in $^{45}Ca^{2+}$ loading, ATPase activity, or the coupling ratio between loading and ATPase activity in treated versus control rabbits (Table II). There was no significant change in $^{45}Ca^{2+}$ loading or ATPase activity with increasing pathology score. Similarly, there was no difference between the treated and control groups in the amount of calcium pump protein in cardiac microsomal fractions determined by densitometry of Coomassie-stained gels (Table II). In these same microsomal fractions, doxorubicin treatment did not alter activities of either the sarcolemmal Na–K pump or the mitochondrial H^+-ion pump

Table II. Characterization of Microsomal Fractions from Treated and Control Rabbits

	Control	Treated	
Protein yield (%)	1.09±0.06 (19)	1.36±0.06 (20)	$P = 0.01$
Pathology score	0.15±0.03 (19)	1.36±0.13 (20)	
CaMg ATPase (nmol · mg^{-1} · min^{-1})	192±10 (19)	179±9 (20)	NS
Ca loading (nmol · mg^{-1} · min^{-1})	155±12 (19)	145±12 (20)	NS
Coupling ratio	0.87±0.10 (19)	0.83±0.06 (20)	NS
NaK ATPase (nmol · mg^{-1} · min^{-1}) (SL)	171±17 (9)	146±26 (9)	NS
Mg ATPase (nmol · mg^{-1} · min^{-1}) (mito)	563±43 (7)	665±39 (7)	NS
100 kD band (densitometry)	Ratio treated/control	0.88±0.05 (13)	NS

Parentheses indicate *n*.

(Table II). All activities are expressed relative to total protein. Thus, despite the small difference in protein yield in microsomal fractions from treated versus control rabbits, these fractions appear very similar with respect to the above assays.

Ryanodine binding scatchard analysis. To screen for differences in either B_{max} or K_d, ryanodine binding was measured in cardiac microsomal fractions at a subsaturating concentration of ryanodine (10 nM). Specific binding at this single concentration was markedly lower in the treated versus the control rabbits (Fig. 2). Complete binding isotherms using pooled samples from different treated or control rabbit hearts revealed that the difference noted above was due to a significantly lower B_{max} without a significant change in K_d (Fig. 3). No difference in ryanodine binding between treated and control was seen after short courses (two to three doses) of doxorubicin in the rabbit model (not shown).

Doxorubicin sensitivity of ryanodine binding. To determine whether chronic doxorubicin exposure shifted the sensitivity to in vitro doxorubicin exposure, microsomal fractions from treated and control hearts were exposed to increasing doxorubicin concentrations at pCa 6.8. At this suboptimal pCa, doxorubicin will enhance binding by shifting the calcium sensitivity (28). At suboptimal pCa there was no difference in the doxorubicin concentration required to reach half maximum for ryanodine binding between control and treated rabbits (Fig. 4).

Calcium sensitivity of ryanodine binding. To determine if there was a shift in the calcium sensitivity of the calcium release channel in doxorubicin cardiomyopathy analogous to the shift in the calcium sensitivity for ryanodine binding seen with in vitro doxorubicin exposure, pCa curves were determined for treated and control cardiac microsomal fractions (Fig. 5). There was a small leftward shift in the pCa_{50} in the treated versus control rabbits that was statistically significant. The apparent difference at very low pCa (i.e., 8) in the treated versus the control rabbits was not statistically significant.

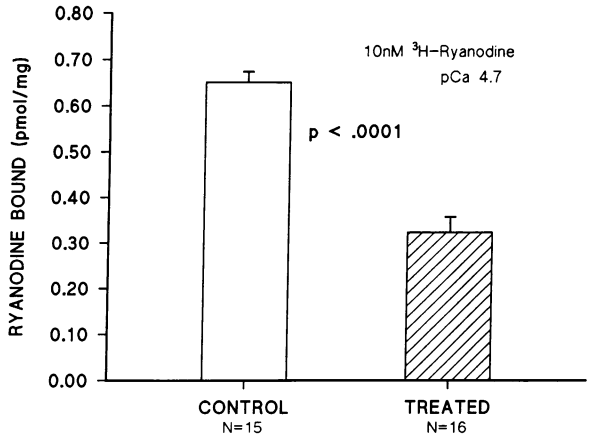

Figure 2. The effect of chronic doxorubicin treatment on [^{3}H]-ryanodine binding. Binding was determined at a single point on the binding isotherm for individual rabbits to detect potential differences in either B_{max} or K_d. Microsomal fractions from each rabbit (N) were assayed in triplicate with 10 nM [^{3}H]ryanodine in the presence (nonspecific binding) or absence (total binding) of an 100-fold excess of unlabeled ryanodine. Vesicle-bound [^{3}H]ryanodine was separated by ultracentrifugation. Values shown are mean±SE for the specific binding. The mean pathological score for the treated rabbits was 1.37 (moderate) in this figure.

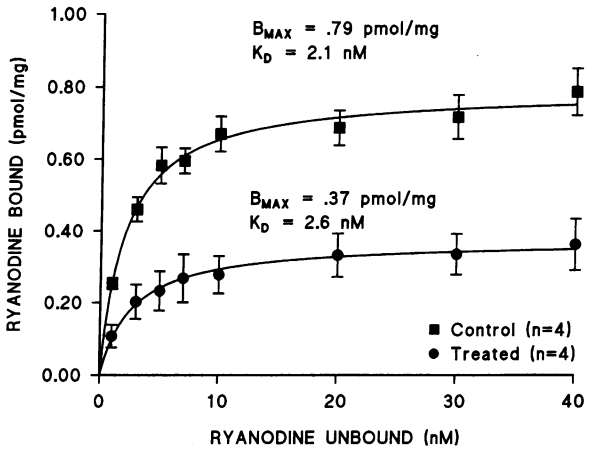

Figure 3. The effect of chronic doxorubicin treatment on [^{3}H]-ryanodine binding. Microsomal fractions were pooled to obtain treated samples (n = 4) and control samples (n = 4) of sufficient volume to run a binding curve on each sample. Nonspecific and total ryanodine binding at eight different ryanodine concentrations, in duplicate or triplicate, were determined for each of the pooled samples. The mean±SE of the specific binding for the treated (n = 4) and control (n = 4) samples is shown. Binding isotherms were generated using the computer program Graphpad. These curves were then converted to Scatchard plots to determine B_{max} or K_d. Nonspecific binding was identical for the control and treated rabbits. The mean pathological score for the treated rabbits was 1.3 (moderate) in this figure.

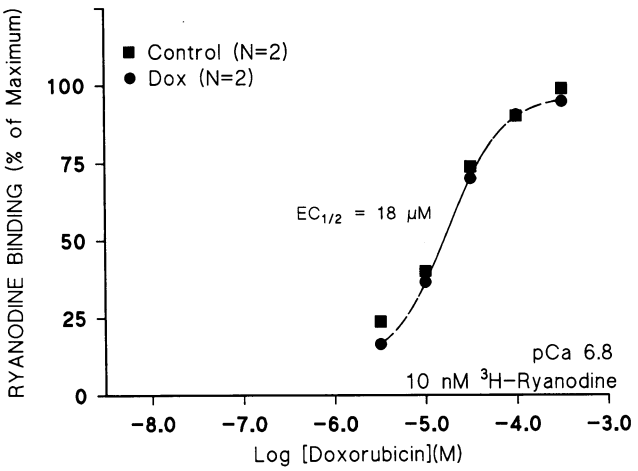

Figure 4. The effect of chronic doxorubicin treatment on in vitro doxorubicin sensitivity of ryanodine binding. To determine if chronic treatment with doxorubicin alters the properties of the calcium release channel, [^{3}H]ryanodine binding in two microsomal samples from treated and control hearts were compared at suboptimal calcium concentration (pCa 6.8) in the presence of five different doxorubicin concentrations. Each assay was performed in triplicate. The curves for control and treated rabbits represent the mean specific binding (percent of maximum) for the two samples, plotted as a function of the logarithm of the doxorubicin concentration. Maximum specific binding was 0.75 pmol/mg for control and 0.30 pmol/mg for treated hearts. Doxorubicin increased specific binding in the control hearts as expected from prior in vitro studies (not shown). Doxorubicin increased specific binding in the doxorubicin-treated hearts, with an $EC_{1/2}$ of 18 μM, which was similar to the response in the control hearts. The mean pathological score for treated rabbits was 1.6 (moderate) for this figure.

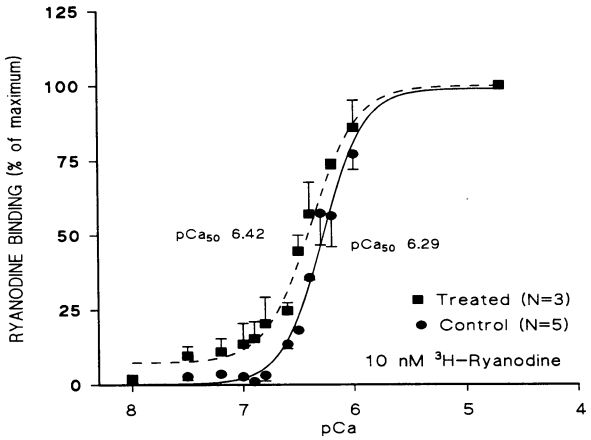

Figure 5. The effect of chronic doxorubicin treatment on the Ca^{2+} sensitivity of ryanodine binding. To determine if chronic treatment with doxorubicin alters the properties of the calcium release channel, pCa curves were compared for treated and control hearts. Cardiac microsomal fractions from control (●) and treated (■) rabbits were incubated with [^{3}H]ryanodine in the presence of variable EGTA concentrations. Specific [^{3}H]ryanodine bound (percent of maximum binding) is shown as a function of pCa. Maximum specific binding was 0.71±0.01 pmol/mg for these control rabbits and 0.28±0.05 pmol/mg for these treated rabbits ($P < 0.01$). The control curve is a composite of assays performed on five different microsomal samples. The treated curve is a composite of assays performed on three different microsomal samples. SE bars are shown for points where $n > 2$. The pCa_{50} for the control rabbits was 6.29±0.04 and that for the treated rabbits was 6.42±0.05 ($P = 0.05$, Mann-Whitney). The mean morphological score for treated rabbits was 1.6 (moderate) for this figure.

Correlation of pathology score with functional data and binding data. To correlate the severity of the cardiomyopathy as determined by the pathology score with other measurements, the treated rabbits were divided into mildly affected (pathology score < 1), moderately affected (pathology score 1–2), and severely affected (pathology score > 2). As demonstrated in Table I and Fig. 6, developed tension at 0.5 Hz decreased as the pathology score increased. Ryanodine binding also decreased as the pathology score rose. The lines fitting the data for developed tension and binding are nearly identical (Fig. 6).

Estimate of calcium release channel by anti-calcium release channel antibody. Western blot analysis of cardiac *homogenates* enabled us to demonstrate that the decrease in calcium release channel in treated versus control rabbit microsomes was not an aberration referable to subcellular fractionation of normal and treated rabbits (Fig. 7).

Western blot analysis of cardiac *microsomal* fractions demonstrated qualitatively lower amounts of immunoreactive calcium release channel in the treated rabbits compared with the controls judged by intensity of color indicator (not shown).

Comparison of effects on skeletal muscle. To determine whether the effect of doxorubicin treatment on sarcoplasmic reticulum was specific to cardiac muscle, skeletal muscle microsomal fractions were isolated from four pairs of doxorubicin-treated and control rabbits. Despite a marked decrease in the ryanodine binding in these cardiac microsomal fractions, there was no difference observed in ryanodine binding in the skeletal microsomal fractions compared with their controls (Fig. 8).

Using the anti-calcium release channel antibody that cross-reacts with both cardiac and skeletal calcium release channel, the amount of calcium release channel in the skeletal microsomal fractions from treated and control rabbits was qualitatively similar, despite a significant decrease in calcium release

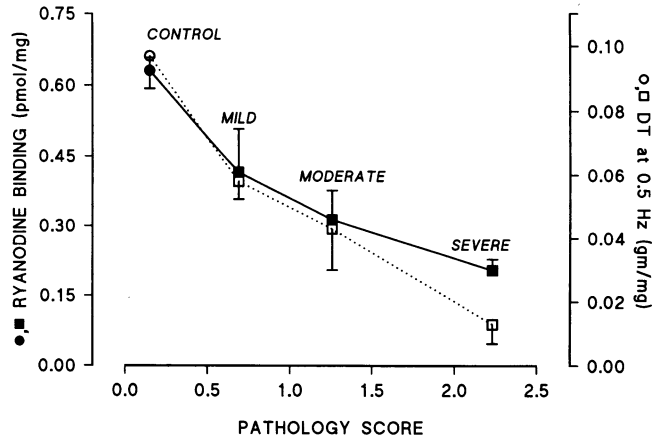

Figure 6. Comparison of ryanodine binding (*solid symbols*) (pmol/mg) and developed tension (*DT*) (*open symbols*) (g/mg at 0.5 Hz) as a function of cardiomyopathy severity. Values represent mean±SE.

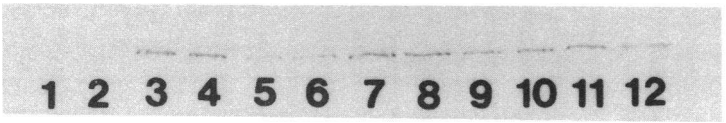

Figure 7. Representative Western blot of homogenates using calcium release channel–specific antibody. Each sample is run in duplicate. Samples from rabbits with different degrees of cardiomyopathy by EM score were compared with their paired controls. Lanes *1* and *2* = severe (EM score = 2.4; ryanodine binding = 0.19 pmol/mg); lanes *5* and *6* = moderate (EM score = 1.3; ryanodine binding = 0.28 pmol/mg); lanes *9, 10* = mild (EM score = 0.9; ryanodine binding = 0.59 pmol/mg). Lanes *3, 4, 7, 8, 11,* and *12* represent the controls.

channel in the cardiac preparations from those treated rabbits (not shown).

Doxorubicin and doxorubicinol concentrations. The atrial tissue concentration of doxorubicin was 0.42±0.10 ng/mg wet tissue weight and of doxorubicinol was 0.07±0.01 ng/mg wet tissue weight in treated rabbits. This corresponds to a doxorubicin concentration of ~ 1 μM assuming that 1 kg of tissue contains ~ 1 liter of fluid.

Discussion

Our studies demonstrate that the cardiomyopathy associated with chronic doxorubicin exposure is accompanied by a decrease in the amount of calcium release channel of the sarcoplasmic reticulum detected by a decrease in radioligand binding using the specific high affinity ligand ryanodine (Fig. 2) and by a reduction in immunoreactive calcium release channel (Fig. 7). Further, the decrease in amount of calcium release channel correlates with the severity of the cardiomyopathy scored by the microscopic criteria of Billingham (15) and with the decrease in developed tension in atrial strips from the doxorubicin-treated rabbits (Fig. 6). This decrease in the calcium release channel was initially detected and has been best characterized in the sarcoplasmic reticulum–enriched microsomal fractions. These fractions from treated and control rabbits are otherwise equivalent with respect to amount of calcium pump protein of the sarcoplasmic reticulum, including calcium-dependent ATPase activity and ATP-dependent oxalate-stimulated calcium loading activity (Table II). The decrease in calcium release channel can be detected in both the microsomal fraction and the homogenate of treated rabbits by Western blot analysis. Therefore, the decrease in calcium release channel detected in the microsomal fractions of treated rabbits is not referable to selective loss of the terminal cisternae (the calcium release channel–rich region of the sarcoplasmic reticulum) during subcellular fractionation. In addition, the calcium release channels present in the treated rabbits do not appear significantly altered compared with the channels of the control rabbits with regard to: K_d for ryanodine binding (Fig. 3) or sensitivity of ryanodine binding to doxorubicin added in vitro (Fig. 4). There was a small difference in pCa_{50} for ryanodine binding, with the calcium release channel from treated rabbits requiring less calcium for binding (Fig. 5). Finally, the decrease in calcium release channel was specific to cardiac muscle, leaving skeletal muscle essentially unaffected (Fig. 8).

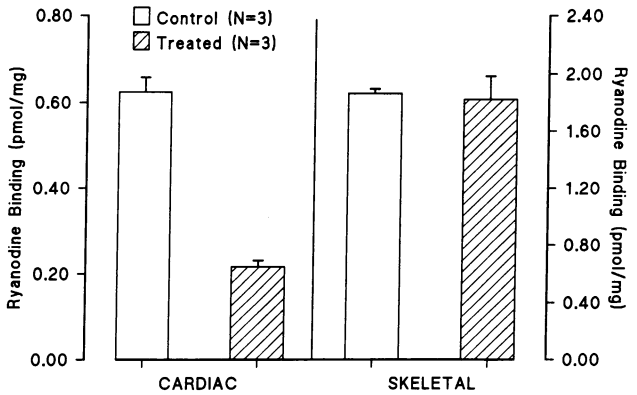

Figure 8. Ryanodine binding in cardiac vs. skeletal microsomal fractions from control and treated rabbits. [^{3}H]ryanodine binding in microsomal preparations from treated (n = 3) and control rabbits (n = 3) was determined in 10 nM ryanodine for both cardiac and skeletal muscle. Values represent the mean±SE of three samples in triplicate. The mean pathologic score for treated rabbits was 1.83 (moderate) in this figure.

This study is unique in several aspects. The first is the use of pair-fed controls. Since various nutritional deficiencies, such as selenium deficiency, are known to cause their own myopathic changes (48), we felt it was crucial to have controls that were better matched in nutritional status. Second, this study is a multilevel study correlating changes in morphology scored by light and electron microscopy, changes in intact muscle function, and changes in subcellular functions resulting from chronic doxorubicin exposure. Finally, it is one of the few studies to evaluate function of various pumps and channels in the chronic setting and the only one to evaluate the calcium release channel.

The results support the hypothesis that the sarcoplasmic reticulum is effected early in the doxorubicin cardiomyopathy as suggested by the sarcotubular dilation noted on histopathology (Fig. 1) (15, 16). It appears to be a specific effect on the calcium release channel of the sarcoplasmic reticulum and does not appear to affect the calcium pump protein of the sarcoplasmic reticulum. The decrease in ryanodine binding would be consistent with several explanations: a decreased amount of calcium release channel; a decrease in high affinity binding secondary to an altered binding site or due to modification of the channel at a regulatory site distant from the ryanodine-binding site. The decrease in receptor was also detected by Western blot analysis using a specific antibody to the calcium release channel. Together these two findings, decreased ryanodine binding and decreased immunoreactive channel, suggest an actual decrease in the amount of channel rather than the presence of an altered channel not detected by either method.

This decrease in amount of calcium release channel resulting from chronic doxorubicin treatment seems, at first glance, to be very different from the increased calcium sensitivity of the channel resulting from in vitro doxorubicin exposure (28). One explanation linking these two observations would be that the decrease in calcium release channel in the chronic model results from a downregulation of the channel in response to

chronic stimulation due to increased calcium sensitivity. We did detect a small shift in calcium sensitivity in the chronic model. The expected shift in pCa_{50} would be small at the measured doxorubicin tissue levels ($\sim$ 1 μM). The shifts in calcium sensitivity may be more marked at peak drug levels after infusion.

Although the changes seen in the calcium release channel of the sarcoplasmic reticulum are not proven to be causal for the doxorubicin cardiomyopathy, the correlation with degree of myopathy and the agreement with prior morphological and in vitro studies suggesting the calcium release channel might be a target make this plausible. Mechanisms for doxorubicin toxicity suggested by earlier studies include: free radical generation and lipid peroxidation (49–52), reactive sulfhydryl groups (53, 54), binding to channel regulatory sites (25, 28, 31, 32), or inhibited mRNA/protein synthesis (55–58). The results to date have been conflicting, possibly complicated by the potential of two different mechanisms, one for acute toxicity and one for the late (chronic) toxicity; or by the potential of different mechanisms during therapeutic dosing versus much higher dosing; or by different mechanisms for doxorubicin toxicity in various in vitro conditions. Furthermore, some mechanisms, such as inhibition of mRNA synthesis, might be expected to be less specific for the calcium release channel than direct interactions with the calcium release channel. Therefore, understanding the mechanism behind the apparent decrease in calcium release channel may also help elucidate the degree of specificity of the sarcoplasmic reticulum as an early site of injury.

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