Effects of In Vitro and In Vivo Exposure to Doxorubicin (Adriamycin) on Caffeine-Induced Ca^{2+} Release from Sarcoplasmic Reticulum and Contractile Protein Function in 'Chemically-Skinned' Rabbit Ventricular Trabeculae

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ABSTRACT—Doxorubicin is an anthracycline antibiotic that is used widely as a chemotherapeutic agent. However, the usefulness of this agent is limited due to its cardiotoxic effects. The mechanisms associated with this cardiotoxicity remain essentially unknown, despite numerous studies describing a range of structural and functional abnormalities. The purpose of the present study was to determine the in vivo and in vitro effects of doxorubicin exposure on sarcoplasmic reticulum (SR) Ca^{2+} -content and contractile protein function. The Ca^{2+} -content of SR is shown to have a biphasic response to in vivo and in vitro doxorubicin exposure that is time- and dose-dependent. In vitro doxorubicin exposure initially reduces the SR Ca^{2+} -content, but the predominant action to block the SR Ca^{2+} -release channel increases SR Ca^{2+} -content within 60 min. Similar results are observed with in vivo doxorubicin exposure: it leads to Ca^{2+} -overload. These data are consistent with the view that doxorubicin acts in a similar manner to ryanodine and results in cardiomyopathy due to Ca^{2+} -overload.

Keywords: Doxorubicin, Sarcoplasmic reticulum, Myofilament, Calcium sensitivity, Ventricular trabeculae (rabbit)

Doxorubicin is an anthracycline antibiotic used commonly for the treatment of a number of human malignancies including solid and hemopoietic cancers. Its clinical usefulness, however, is limited by its cardiotoxicity. The clinical manifestations of anthracycline-induced cardiotoxicity can be divided into two categories: 1) in vitro effects, manifested by atrial and ventricular arrhythmias (1), and 2) a chronically developing cumulative dose-dependent cardiomyopathy (2). The mechanism by which this cardiotoxicity can occur is essentially unknown despite numerous studies describing a wide range of structural and functional abnormalities.

There is compelling evidence that anthracyclines alter the function of cardiac sarcoplasmic reticulum (SR). In a chronic rabbit model, doxorubicin-induced cardiomyo-

pathy was associated with a decrease in SR Ca^{2+} -release channel density (3). Since SR Ca^{2+} -release is thought to represent the predominant source of activator Ca^{2+} for myocardial contraction (4), any decrease in the ability of the SR to release Ca^{2+} could be responsible for the contractile dysfunction in doxorubicin-induced cardiomyopathy. If SR Ca^{2+} -release channels are the main targets of anthracyclines, depressed contractility should also be an early manifestation of cardiotoxicity. Indeed, myocardial contractility was depressed in the early phase after acute administration of high doses of doxorubicin in humans (5). In contrast, doxorubicin produced persistent positive inotropic effects in isolated cardiac preparations (6) and increased left ventricular systolic and diastolic function 4 and 24 hr after the treatment of tumors in patients with normal ventricular function (7).

The purpose of the present study was determine the

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reason for these discrepant findings by studying the effects of both in vitro and in vivo exposure to doxorubicin on the function of SR and contractile proteins determined under the same experimental conditions using chemically-skinned rabbit ventricular trabeculae.

MATERIALS AND METHODS

The rationale for solution composition, the method of calculating free ion levels and ionic strength, the choice of ion binding constants for the various ligands, precautions for ethylene glycol bis (β -aminoethyl ether) N,N,N,N' -tetraacetic acid (EGTA) purity, calcium contamination determination, the measurement of pH, and other details of our experimental protocol have been described elsewhere (8, 9). Experiments were carried out at room temperature (20–22°C).

Isolation of ventricular trabeculae

Trabeculae were isolated and prepared in the same manner from doxorubicin-treated and untreated rabbits for the in vitro experiments. Male New Zealand White rabbits were killed with a lethal dose of pentobarbital sodium (200 mg/kg), the heart was excised rapidly and a trabecula was isolated from the right ventricle. Trabeculae of appropriate size (<200 μ m diameter, 2- to 3-mm-long) were commonly situated close to the tricuspid valve annulus or occasionally at the right ventricular apex.

Mounting for isometric force measurement

The preparation was mounted for isometric force measurement by snaring at either end (using nylon monofilaments) between two stainless steel tubes (200- μ m, o.d. and 100- μ m, i.d.). The tubes were fixed to a force transducer at one end (model AE 875; Akers, Horton, Norway) and a fixed point on the other. The whole mount was carried on a modified Narishige MM3 micro-manipulator, which permitted adjustment of the tube separation (and hence muscle length). Sarcomere length

was measured by differential interference contrast light microscopy and set to 2.1–2.2 μ m.

Solution change and chemical skinning procedure

Once the muscle had been mounted, it was exposed to a 'relaxing' solution (solution B, Table 1) containing saponin (50 μ g/ml) for 20–30 min. This permeabilises the sarcolemma leaving the SR and myofilaments functionally intact. Solutions were contained in a series of wells (each containing 5 ml of fluid) cut into a perspex block and which were continuously stirred with a small rotating stainless steel paddle. A solution change was made by moving a perspex block containing different solutions with two stepper motors, under computer control, which lowered the perspex block, moved it horizontally and then raised it to immerse the preparation to a different bathing solution.

Doxorubicin administration model

Male New Zealand White rabbits (2.5–3 kg) were given i.v. doxorubicin (1 mg/kg) twice weekly for 8 weeks via the marginal ear vein (control animals were given saline injections). Animals were assessed three times weekly for signs of toxicity and distress using the scoring system proposed by Morton and Griffiths (10). Specific features noted were anorexia, weight loss, diarrhea, mucosal ulceration, conjunctivitis, alopecia and nasal discharge. If the animal was scored above a threshold level (total score of 10) on this scale, it was euthanized. On completion of the 8-week course of injections, the animals developed chronic heart failure over the following 2 to 4 weeks (11).

Hemodynamic assessment

Hemodynamic assessment was done 2 to 4 weeks after completion of doxorubicin injections. Echocardiography was used to measure left ventricular end-diastolic diameter (LVEDd) and left ventricular ejection fraction (LVEF) in all animals. Invasive hemodynamic measurements were made in a conscious animal 24 hr after im-

Table 1. Composition of solutions at pH 7.0

Solution	K ⁺ * (mM)	Mg ²⁺ † (mM)	Ca ²⁺ (mM)	pCa	ATP (mM)	CrP (mM)	Na ⁺ † (mM)	EGTA (mM)	HEPES (mM)
A	140	7.0	0.02 ^a	7.29	5.0	15.0	40.0	0.2 ^b	25.0
B	140	7.0	0.02	9.03	5.0	15.0	40.0	10.0	25.0
C	140	7.0	10.0	4.20 [§]	5.0	15.0	40.0	10.0	25.0

CrP, creatine phosphate. *Potassium ions added as KCl and KOH. †Magnesium added as 1.0 M MgCl₂ (free Mg²⁺ = 2.1–2.5 mM in all solutions). ‡Sodium ions from the salts Na₂ ATP and Na₂ CrP.

§An additional 0.1 mM CaCl₂ was added to aliquots of solution C to yield "full activating" solution (pCa 3.98). Total chloride concentration varied from about 110 to 120 mM. ^aEstimated Ca²⁺ concentration level. ^b1,6 diaminoethane N,N,N,N' -tetraacetic acid (9.8 mM) was added to this solution to maintain equivalent ionic strength.

plantation (under general anesthesia) of a left ventricular catheter (via the carotid artery) and a thermodilution cardiac output catheter (placed in the descending aorta via the femoral artery). Measurements of left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and cardiac output were made at baseline and after a bolus injection of saline into the left ventricle (15 ml/kg over 1 min). All saline-treated animals (Sal) were assessed invasively, while invasive data were obtained for only seven doxorubicin-administrated animals (Dox). Twenty animals were started on doxorubicin, but only twelve completed the protocol. Six

animals with high distress scores were euthanized during the course of doxorubicin injections, and two died while placing the catheters for invasive hemodynamic assessment.

SR function after in vitro exposure of doxorubicin

Figure 1A illustrates the protocols used to assess in vitro effects of doxorubicin on caffeine-induced contraction amplitude in a saponin-skinned trabecula isolated from a Sal animal. The preparation was exposed to 200 nM Ca^{2+} in solution A (Table 1). This concentration is below the threshold for myofilament activation but sufficient to allow the SR to accumulate Ca^{2+} . Calcium-release from the SR was triggered by the application of 10 mM caffeine. The amplitude of the resultant caffeine-induced contraction was used to indicate releasable Ca^{2+} -content of the SR. When a reproducible series of caffeine-induced contractions had been established, at constant $[\text{Ca}^{2+}]$ (200 nM) and loading time (120 sec), the preparation was exposed to doxorubicin. The dose- and time-dependency of SR Ca^{2+} -loading and release to doxorubicin exposure were routinely assessed in its presence.

SR function after administration of doxorubicin

Figure 1B illustrates the protocol used to assess the effects of in vivo doxorubicin exposure on caffeine-induced contraction amplitude. The protocol is similar to that described above for the in vitro exposure, except that the preparations were exposed to different loading $[\text{Ca}^{2+}]$ s (rising by 50 nM increments from 150–300 nM) and loading times (ranging from 15–150 sec in random order). Furthermore, SR function was assessed both in the presence and absence of doxorubicin.

Myofilament function after in vitro exposure to and administration of doxorubicin

After assessing SR function, the preparation was exposed to Triton X-100 (1% [vol./vol.]) for 20–30 min. This process disrupts all cellular membranes and only the myofilaments are functionally retained. The preparation was activated to produce tension by increasing the free $[\text{Ca}^{2+}]$ in the solution. Myofilament Ca^{2+} -sensitivity was assessed by altering the $[\text{Ca}^{2+}]$ over the appropriate range (test solutions were made by mixing solutions B and C, Table 1). Each test $[\text{Ca}^{2+}]$ was applied after prior equilibration of the preparation in solution A in order to activate the muscle as rapidly and uniformly as possible.

Data collection and analysis

The output of the tension transducer was displayed on a chart recorder (Astromed Dash 8; Astromed, West Warwick, RI, USA), after being pre-amplified and filtered at 25 Hz. Simultaneously, the signal was digitized

Fig. 1. Experimental protocol for assessing SR Ca^{2+} -loading. **A:** Experimental trace demonstrating the effects of in vitro doxorubicin exposure in a saponin-treated trabecula. The trabecula was loaded in a standard way by immersion in a weakly calcium buffered solution (free calcium concentration, 200 nM) for 2 min. The preparation was then activated by exchanging the bathing solution for one containing caffeine (10 mM) as indicated by the arrow heads below the trace. Once a stable response had been achieved, 5×10^{-5} M doxorubicin was added to the bathing solution (indicated by the solid bar above the trace). Contraction amplitude was taken as an indication of the calcium content of SR. **B:** Experimental trace demonstrating the effects of in vivo doxorubicin exposure in a saponin-treated trabecula. Arrow heads represent a 5-sec application of 10 mM caffeine. A train of three control caffeine-induced contractions is followed by the test loading of $[\text{Ca}^{2+}]$ for various periods (15–150 sec) arranged in random order. The preparation is then returned to the standard control conditions for a train of five caffeine-induced contractions before starting the protocol again at a higher loading $[\text{Ca}^{2+}]$.

at appropriate rates using a commercial data acquisition system (MacLab; AD Instruments Pty, Ltd., New South Wales, Australia) and commercially available Chart software. The relationship between $[Ca^{2+}]$ and tension in our experiments is well described by the Hill equation. Curves were fitted by a least squares fitting procedure after Levenberg and Marquadt (12). This yields values for $K_{1/2}$, the reciprocal of the $[Ca^{2+}]$ producing half-maximum calcium-activated force, and h , the Hill coefficient. To avoid distortion produced by pooling experimental data, curves were fitted on an individual basis. Data are presented as the means of such shifts and $K_{1/2}$ s. The Hill coefficients are treated in the same way. Maximum calcium-activated force (C_{max}) was measured using solution C (Table 1) with excess calcium added (providing a final pCa of 4.0). In the protocol to assess SR function after administration of doxorubicin, caffeine-induced contractions are expressed as a percentage of the first C_{max} measured after exposure to Triton X-100. In the protocol to assess SR function after in vitro exposure to doxorubicin, caffeine-induced contractions are expressed as a percentage of the control activations made immediately prior to doxorubicin exposure. Collected data are expressed as the mean $\pm$ S.D. Statistical analyses were performed by paired and non-paired t -tests. Significance was taken at the 5% level.

RESULTS

In vitro effects of doxorubicin on SR function

Exposure of saponin-skinned trabeculae isolated from

Fig. 2. Graph showing the effective concentration-response curve to doxorubicin in saponin-treated trabeculae. The vertical axis displays the relative force produced and the horizontal axis, doxorubicin concentration. Data are expressed as the mean $\pm$ S.D. and are normalized to the initial caffeine-induced tension response. The numbers next to the data points are numbers of experiments contributing to each point. The data are fitted by the Hill equation with $h=1$.

a stock (intact) animal to doxorubicin caused a decrease in the caffeine-induced contraction. Figure 1A demonstrates this in vitro effect of 5×10^{-5} M doxorubicin. The dose-dependency of this effect is shown in Fig. 2. Doxorubicin exhibits a classical sigmoidal concentration-response relationship where the concentration giving rise to a 50% reduction in the caffeine-induced contraction

Fig. 3. Experimental trace demonstrating the effect of more prolonged exposure to doxorubicin. The experimental conditions were similar to those described in Fig. 1A except that the break in the trace (indicated by the two slanted lines) represent 60 min of exposure to 5×10^{-5} M doxorubicin. The preparation displays spontaneous tension oscillations and, despite this, the amplitude of the caffeine-induced contractions was increased.

Fig. 4. Graph demonstrating the effect of 5×10^{-5} M doxorubicin on myofilament calcium sensitivity and force production. The data are expressed as the mean $\pm$ S.D. ($n=3$) and are scaled to the control maximum calcium-activated force. The vertical axis displays the relative force produced and the horizontal axis, calcium concentration. The solid or open circles are the calcium-tension response before or after doxorubicin exposure, respectively. The symbols have been displaced slightly for clarity. The data are fitted by the Hill equation.

(EC_{50}) was 6.9×10^{-5} M. Doxorubicin also has time-dependent effect that is not illustrated here.

Figure 3 demonstrates the effect of a more prolonged exposure to 5×10^{-5} M doxorubicin on the caffeine-induced contracture. The protocol is similar to that in Fig.

1A except that doxorubicin is applied at constant concentration (5×10^{-5} M) for 60 min, as indicated by the break in the trace. Although the caffeine-induced contractions were not reproducible, the amplitude of the caffeine-induced contraction tended to increase. Moreover, the preparations displayed spontaneous tension oscillations. It is important to note that similar results were obtained when doxorubicin was removed during the caffeine-induced contractures after the more prolonged exposure ($n=6$ in both cases, data not shown).

In vitro effects of doxorubicin on myofilament function

By studying maximum and sub-maximum calcium-activations in the Triton X-100 treated preparations, we are able to determine the effects of in vitro doxorubicin exposure on myofilament function. Maximum calcium-activated force fell from 100% (by definition) to $95.4 \pm 3.8\%$ ($n=3$, ns) on exposure to doxorubicin (5×10^{-5} M). Figure 4 shows the relation between tension and $[Ca^{2+}]$ obtained from a range of sub-maximal calcium-activations in three preparations. Sub-maximal calcium-activations are expressed relative to C_{max} for that series of activations. The best-fit curves obtained are superimposable, indicating that in vitro exposure to doxorubicin produced no significant alteration in myofilament calcium sensitivity (pCa_{50} , 5.39 vs 5.38) and little change in C_{max} .

Administration of doxorubicin in vivo

General manifestations: Animals injected with doxo-

Fig. 5. Graphs demonstrating SR Ca^{2+} -loading at four $[Ca^{2+}]$ s (A, 150 nM; B, 200 nM; C, 250 nM; and D, 300 nM) in preparations from doxorubicin- and saline-treated rabbits. The vertical axis displays the amplitude of the caffeine-induced contracture expressed relative to maximum calcium-activated force obtained for each preparation after Triton X-100 treatment. The horizontal axis displays the duration of the loading period at each $[Ca^{2+}]$. Data are expressed as the mean $\pm$ S.D. Doxorubicin-treated animals (solid circles, $n=12$) demonstrate greater SR Ca^{2+} -loading than saline-treated animals (open circles, $n=7$) only at higher $[Ca^{2+}]$ s. * $P < 0.05$ and ** $P < 0.01$ vs corresponding control values.

Fig. 6. Graph demonstrating SR Ca^{2+} -loading at 250 nM $[\text{Ca}^{2+}]$ for doxorubicin injected rabbits separated on the basis of ejection fraction. The figure is annotated in a similar way to Fig. 5. Doxorubicin-treated animals with EF < 45% (solid circles, $n=4$) had enhanced Ca^{2+} -loading compared with similarly treated animals with EF > 45% (solid squares, $n=8$) and saline-treated animals (open circles, $n=7$). Data are expressed as the mean $\pm$ S.D.

rubricin (Dox animals) had significantly lower body weights than the saline-treated controls (Sal animals) (3.02 ± 0.37 vs 3.68 ± 0.25 kg, $P < 0.001$; Dox vs Sal). A significant proportion of Dox animals had scrotal edema (Dox, 75% vs Sal, 0%), tachycardia of >240 beats/min (Dox, 41.6 vs Sal, 0%) and tachypnoea >50 breaths/min (Dox, 41.6 vs Sal, 0%). The relative wet weights of liver and lung to body weight in Dox animals were not different from those of saline-treated controls (data not shown).

Echocardiography: Left ventricular end-diastolic diameter was significantly greater in Dox animals (1.63 ± 0.11 vs 1.50 ± 0.16 cm, $P = 0.048$), although 50% of these animals had values that fell within the control range (1.2–1.6 cm). Left ventricular ejection fraction was significantly lower in Dox animals ($54 \pm 9.5\%$ vs $70.5 \pm 6.5\%$, $P < 0.01$). However, again there was considerable overlap of values (Dox range 35–70%, Sal range 60–80%).

Left ventricular pressures: Left ventricular systolic pressure was not significantly different in Dox animals (93.4 ± 11.8 mmHg) compared with Sal animals (101.5 ± 14.1 mmHg). Also, there was no significant difference in left ventricular end-diastolic pressure between the groups at baseline (6.2 ± 4.1 vs 5.1 ± 3.4 mmHg) or in response to a fluid challenge (11.7 ± 7.6 vs 11.5 ± 7.2 mmHg).

Cardiac output: Cardiac output tended to be slightly lower in Dox animals (292 ± 71.2 ml/kg/min) than Sal animals (332 ± 53.5 ml/kg/min; ns). The response of

Fig. 7. Graph demonstrating the effect of in vivo doxorubicin exposure on myofilament calcium sensitivity and force production. The data are scaled to the control maximum calcium-activated force. The vertical axis displays the relative force produced and the horizontal axis the calcium concentration. Doxorubicin-treated animals (solid circles, $n=10$, mean $\pm$ S.D.) show an insignificant decrease in myofilament calcium sensitivity when compared with saline-treated animals (open circles, $n=6$). The $[\text{Ca}^{2+}]$ required for half-maximal activation (pCa_{50}) being slightly lower and the Hill-coefficient being slightly higher (Dox vs Sal: pCa_{50} , 5.49 ± 0.09 vs 5.55 ± 0.1 ; h , 3.95 ± 0.1 vs 3.56 ± 0.65) in preparations obtained from doxorubicin-treated animals.

cardiac output to fluid challenge was also not significantly different (31.2 ± 10.1 vs 35 ± 16.8). Once again the range of cardiac outputs in Sal animals was smaller than that in Dox animals (211–344 vs 303–387 ml/kg/min), indicating that not all Dox animals had significant LV dysfunction.

SR function: Figure 5 shows curves demonstrating SR Ca^{2+} -loading at different $[\text{Ca}^{2+}]$ s. There was no significant difference in amplitude of caffeine-induced contractions between Dox and Sal animals at the lower loading $[\text{Ca}^{2+}]$ s (150–200 nM) (Fig. 5: A and B). However, at 250 nM loading $[\text{Ca}^{2+}]$, the Dox animals demonstrated larger caffeine-induced contractions at both short and long loading period than Sal animals (Fig. 5C). At 300 nM loading $[\text{Ca}^{2+}]$, caffeine-induced contractures were larger in Dox animals at longer loading periods, but not significantly different at shorter loading periods (Fig. 5D).

Animals treated with doxorubicin could be separated on the basis of the left ventricular ejection fraction (EF). Animals with an EF of less than 40% ($n=4$) demonstrated larger caffeine-induced contractions at 250 nM loading $[\text{Ca}^{2+}]$ compared with Sal animals and Dox animals with a normal EF (Fig. 6). This difference between Dox animals with and without LV dysfunction was not observed at other loading $[\text{Ca}^{2+}]$ s.

High levels of SR Ca^{2+} -loading (indicated by the larger

caffeine-induced contractions) were associated with an increase in the tendency for spontaneous tension oscillations. Spontaneous oscillations were defined as a spontaneous increase in tension lasting longer than 2–3 sec and with an amplitude 3 times greater than baseline noise. The $[Ca^{2+}]$ ·time product at which these oscillations first appeared was lower in Dox animals than Sal animals (31.6 ± 3.6 vs $39.7 \pm 2.1 \mu M \cdot min$, $P < 0.01$). These oscillations were associated with a fall in the amplitude of subsequent caffeine-induced contractures. This feature can be clearly seen in the SR loading curve at 300 nM $[Ca^{2+}]$ (Fig. 5D).

Myofilament function: There was no significant difference in the cross-sectional area of trabeculae from Dox and Sal animals (0.079 ± 0.008 vs $0.080 \pm 0.007 mm^2$) or maximum Ca^{2+} -activated force per unit cross-sectional area (7.42 ± 3.0 vs $6.08 \pm 2.93 g \cdot wt/mm^2$). Figure 7 shows the relation between tension and $[Ca^{2+}]$ obtained from a range of sub-maximal calcium-activations. Myofilament calcium sensitivity is slightly, but insignificantly, decreased in Dox animals (pCa_{50} , 5.49 vs 5.55).

DISCUSSION

Abnormalities of the SR are characteristic early features of doxorubicin cardiotoxicity (due to intracellular Ca^{2+} -overload) (13), and Holmberg and Williams (14) provided evidence of a specific interaction between the drug and the terminal cisternal regions of cardiac SR. They also proposed that doxorubicin selectively activated a Ca^{2+} efflux pathway in the SR. The main observations of the present study are: 1) in vitro exposure to doxorubicin decreases the amplitude of caffeine-induced contractions and is without significant effect on the myofilament Ca^{2+} sensitivity, and 2) in vivo (chronic) exposure to doxorubicin increases the amplitude of caffeine-induced contractions without significant effects on the myofilaments.

In vitro effects of doxorubicin exposure

Rapid application of caffeine has been employed in this study as a means to determine the steady-state SR Ca^{2+} -content. An inherent limitation with this indirect approach is that the actual amount of Ca^{2+} -release is not easy to determine. Under standard experimental conditions, for studies of this type, the SR is fully loaded and in equilibrium with the bathing solution within 120 sec. Application of 10 mM caffeine releases only 40% of the total SR Ca^{2+} -content (15). However, provided that myofilament Ca^{2+} sensitivity were unaltered, the amplitude of the caffeine-induced contracture does reflect a constant proportion of total SR Ca^{2+} -content (15). The ability to use this approach reproducibly in the same preparation

makes it a very sensitive means of assessing changes in SR Ca^{2+} -content. Since SR Ca^{2+} -content, for any given loading period, is the net result of Ca^{2+} -influx (via the pump) and Ca^{2+} -efflux (via the channel), any factor acting on these processes could alter SR Ca^{2+} -content (16, 17).

In this study, in vitro exposure to doxorubicin caused a rapid reduction in caffeine-induced contracture amplitude (and thus SR Ca^{2+} -content), whereas the larger caffeine-induced contractures observed after a 60 min exposure revealed an increase in SR Ca^{2+} -content. Doxorubicin metabolism occurs through cytoplasmic reductases (18). As the major metabolite of doxorubicin, doxorubicinol, is a potent inhibitor of the SR Ca^{2+} -ATPase pump (19), and this action alone may account for the initial reduction in SR Ca^{2+} -content. However, doxorubicin is also known to promote the open state of the ryanodine-sensitive SR Ca^{2+} -release channel (20) and so both of these effects could have acted to reduce SR Ca^{2+} -content, i.e., reduced Ca^{2+} -influx (via the pump) and continuous Ca^{2+} leak (efflux) from SR (via the channel).

The apparent increase in calcium content of SR after a 60-min exposure is more difficult to explain and a number of mechanisms could be suggested. If doxorubicin acts in a similar fashion to ryanodine (21), an initial increase in open probability of the channel may occur. Low concentrations of or short exposure to ryanodine opens the cardiac SR Ca^{2+} -release channel due to occupation of the high affinity ($K_d \sim 10$ nM) ryanodine sites (22). At high ryanodine concentrations, or longer exposure, the Ca^{2+} -release channel appears to be locked in a closed state (23), possibly through ryanodine binding to low affinity sites. Lai et al. (24) have demonstrated a maximum of one high affinity binding site per tetramer, depending on $[Ca^{2+}]$ and $[ATP]$, and three additional low affinity sites (K_d 1–5 μM). This may reduce Ca^{2+} -efflux from the SR and results in an increase in SR Ca^{2+} -content. If doxorubicin would act like ryanodine, this may provide an explanation for our findings.

In vivo effects of doxorubicin exposure

The severity of heart failure produced by administration of doxorubicin, in this study, was limited by non-cardiac toxic side-effects. Echocardiography demonstrated that only 4 animals in the Dox group had a low ejection fraction ($<40\%$) and an increased LVEDd. Invasive hemodynamic assessment failed to demonstrate significant differences between Dox and Sal animals. This result is in keeping with other studies (employed the same experimental protocol as that of the present study) that also reported a high rate mortality (25) and a wide variation in the degree of LV systolic function in response to a standard dosage regimen of doxorubicin (11).

Despite the lack of significant changes in LV systolic function, there were demonstrable differences in SR function between the groups. Above 200 nM loading $[Ca^{2+}]$, the larger caffeine-induced contractures observed in preparations isolated from Dox animals suggest a higher SR Ca^{2+} -content. This observation is consistent with an increase in total SR Ca^{2+} -content; although we have no evidence that doxorubicin alters SR volume and calsequestrin levels, an increase in SR volume and/or Ca^{2+} -buffering capacity of the SR is one possible mechanism to explain the increased total SR Ca^{2+} -content. Administration of doxorubicin has been shown previously to decrease Ca^{2+} -release channel density and to cause SR dilatation (3). Moreover, SR distension was the earliest morphological change observed in myocardial biopsies from patients receiving anthracyclines (26). Enhanced SR Ca^{2+} -loading was more marked in the animals with low ejection fractions ($<40\%$) and could represent either an adaptation to the cardiomyopathy induced by doxorubicin (27) or to the direct actions of doxorubicin itself. In vivo doxorubicin exposure was also associated with an increased tendency for spontaneous tension oscillations at higher loading $[Ca^{2+}]$ and tends to indicate SR Ca^{2+} -overload.

There was no difference in myofilament force production between preparations isolated from Dox and Sal animals. These observations are consistent with the work of Jensen (28) who showed no reduction in the force produced by papillary muscles isolated from rats chronically treated with doxorubicin.

Possible mechanism of the observed effects of doxorubicin

The 'free radical' hypothesis may provide the most rational explanation for anthracycline-induced cardiotoxicity. Quinone-containing anthracyclines, like doxorubicin, generate superoxide anion radicals by redox cycling of quinones and semi-quinones (the quinone moiety can be reduced to a semi-quinone by single electron donors such as NADPH). Free radical production by doxorubicin requires addition of NADH-dependent respiratory substrates (29) that were not supplied here. Additionally, the non-enzymic mechanism for free radical production requires iron to act as a co-factor and this is unlikely to occur in our experimental solutions due the strong chelation of such cations by EGTA. In our animal model of in vivo doxorubicin exposure, these limitations do not occur. Furthermore, the nature of the caffeine-induced contractures are identical to those observed after 60 min of exposure (suggesting similar cardiotoxic actions after in vitro and in vivo exposure). These data tend to suggest that free radical production is not required for the doxorubicin-induced alterations in SR Ca^{2+} -content

observed in our study.

In summary, the data obtained in the present study support the view that the SR displays a biphasic response to doxorubicin exposure that is time- and dose-dependent. These observations suggest that doxorubicin has analogous actions to that of ryanodine. In vitro doxorubicin exposure initially reduces the SR Ca^{2+} -content by a predominant action on the SR Ca^{2+} -release channel but increases SR Ca^{2+} -content within 60 min after exposure. In vivo doxorubicin exposure increases total SR Ca^{2+} -content and results in Ca^{2+} -overload.

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