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Time-related increases in cardiac concentrations of doxorubicinol could interact with doxorubicin to depress myocardial contractile function

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1 The present study evaluated the time-dependency of acute anthracycline cardiotoxicity by varying the duration of exposure of rabbit isolated atria to doxorubicin and determing changes (1) in contraction and relaxation and (2) in atrial concentrations of doxorubicin and its C-13 hydroxy metabolite, doxorubicinol.

2 Following addition of doxorubicin (175 μM) to atria, contractility (dF/dt), muscle stiffness (resting force, RF) and relaxation (90% relaxation time, 90% RT) were monitored for a 3.5 h period.

3 Doxorubicin (175 μM) progressively diminished mechanical function (decreased dF/dt, increased RF and prolonged 90% RT) over 3 h. Doxorubicinol (1.8 μM), however, failed to produce time-related cardiac dysfunction; it depressed contractile function and increased muscle stiffness during the first 30 min without causing additional cardiac dysfunction during the remaining 3 h of observation. Doxorubicinol had no effect on 90% RT.

4 During treatment with doxorubicin, atria contained considerably more doxorubicin than doxorubicinol (ratio of doxorubicin to doxorubicinol ranged from 778 to 74 at 0.5 and 3 h, respectively). Elevations of doxorubicin and doxorubicinol in atria paralleled the degree of dysfunction of both contraction and relaxation; increases in muscle stiffness, however, were more closely associated with increases of doxorubicinol than doxorubicin.

5 To probe the relation between cardiac doxorubicinol and myocardial dysfunction further, without confounding effects of cardiac doxorubicin, concentration-response experiments with doxorubicinol (0.9–7.2 μM) were conducted.

6 Plots of doxorubicinol concentrations in atria vs contractility indicated that the cardiac concentration of doxorubicinol, at which contractility is reduced by 50%, is five fold lower in doxorubicin-treated than in doxorubicinol-treated preparations. Thus, doxorubicin and doxorubicinol appear to interact to depress contractile function.

7 Cardiac concentrations of both doxorubicin and doxorubicinol, as observed in these studies, were found to stimulate markedly Ca^{2+} release from isolated SR vesicles, but 3 μM doxorubicinol promoted a 15 fold greater release rate than 3 μM doxorubicin.

8 Our observations coupled with the previously reported finding that doxorubicinol inhibits Ca^{2+} loading of SR, suggests that doxorubicinol accumulation in heart contributes to the time-dependent component of doxorubicin cardiotoxicity, through a mechanism that could involve perturbations of Ca^{2+} homeostasis.

Keywords: Doxorubicin; doxorubicinol; cardiotoxicity; sarcoplasmic reticulum; Ca^{2+} release; Ca^{2+} loading; kinetics; dysfunction of contraction and relaxation

Introduction

Myocardial preparations are highly susceptible to the cardiotoxic effects of anthracyclines such as doxorubicin and daunorubicin. *In vitro* studies, while clearly demonstrating the concentration-related nature of the toxicity, also suggest that duration of exposure to an anthracycline may be a major determinant of cardiac dysfunction. Studies in rat Langendorff preparations (Miwa *et al.*, 1986; Pelikan *et al.*, 1986) and in guinea-pig isolated atria (Hagane *et al.*, 1988) showed progressively greater inhibition of cardiac function following doxorubicin treatment during 60 or 180 min observation periods. The above-mentioned studies, however, were not designed to characterize or address the time-dependent component of doxorubicin cardiotoxicity; the relation between exposure time to anthracyclines and acute cardiac dysfunction remains to be investigated.

The demonstration by Olson *et al.* (1988) that cardiac tissue can metabolize doxorubicin to a highly potent cardiotoxin, doxorubicinol, has raised the possibility that anthracycline toxicity over time could be related to this myocardial metabolism. Thus, it is important to determine whether (1) the heart synthesizes sufficient concentrations of doxorubicinol to impair contraction and relaxation and whether (2) the rate of doxorubicinol synthesis parallels the rate of development of myocardial dysfunction. It is also relevant to determine if treatment with doxorubicinol alone produces time-dependent cardiotoxic effects, as would be expected if doxorubicin and doxorubicinol exert toxicities by similar mechanisms. Differing mechanisms of toxicity could result in antagonistic or synergistic interactions between parent anthracycline and metabolite.

To address such issues, our study investigated the relation between time-dependent alterations in atrial contraction and

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relaxation and rates of change of doxorubicin or doxorubicinol in isolated atria treated with either doxorubicin or doxorubicinol. Abilities of these compounds to affect Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles were also compared, to provide additional information about how doxorubicin or its metabolite, doxorubicinol, could impair cardiac function. This paper describes major differences between cardiac effects of doxorubicin and doxorubicinol, which were especially prominent during prolonged exposure to the agents.

Methods

Isolated atria preparations

Experiments were conducted in accordance with the Declaration of Helsinki and the Guide for Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Adult (2.5–3.5 kg) New Zealand white rabbits of either sex were killed by captive bolt discharge to the cranium. Immediately, a median sternotomy was performed, and the heart removed within 30 s. After placing the heart in iced Krebs-bicarbonate buffer (see below), the left atrium was dissected free, and divided into two thin strips (100 mg each); each muscle strip was placed in a thermojacketed, 25 ml, muscle bath (maintained at 30°C) containing Krebs-bicarbonate buffer composition, mM: NaCl 127, $CaCl_2$ 2.5, KCl 2.3, $NaHCO_3$ 25, KH_2PO_4 1.3, glucose 5.6; the buffer was continuously bubbled with 95% O_2 and 5% CO_2 to maintain a pH of 7.4.

The muscle was attached to an isometric force transducer and stretched to a resting tension of 0.5 g. Muscles were electrically stimulated (S88 stimulator, Grass Medical Instruments, Quincy, MA, U.S.A.) with a square wave pulse (3 ms in duration) at a voltage 10% above threshold voltage of the muscle. Atrial strips were stimulated at 1 Hz and allowed to stabilize for 90–180 min. Experiments were conducted with muscles contracting at a frequency of 2 Hz. Cardiac functional variables examined for each muscle preparation were resting force (RF; mg), maximal rate of rise of force (dF/dt; $g\,s^{-1}$), and 90% relaxation time (90% RT; time for peak developed force to decrease by 90%; ms). Variables evaluated were recorded using high speed (100 mm s^{-1}) oscillographic tracings (Gould 4200S oscillographic recorder) and data analyzed using a Buxco Pulsatile Analyzer (Buxco Electronics, Inc., Sharon, CT, U.S.A.).

Experimental design

Atrial effects of doxorubicin or doxorubicinol were determined 30, 90 and 150 and 210 min after addition of doxorubicin (175 μM) or doxorubicinol (1.8 μM) to the bath. Preliminary trials had shown that the above concentration of either agent produced only a slight, but statistically significant depression of contractile function during a 30 min treatment period, thereby allowing sufficient latitude for each agent to express its ability to compromise cardiac function further during the final 3 h of the experiment. Concentration-response relationships to doxorubicinol were investigated by increasing the concentration of doxorubicinol in the bath in a stepwise fashion (0.9, 1.8, 3.6, 7.2 μM) and measuring functional variables 30 min after each increase (i.e., doxorubinicol was added at times 0, 60, 120 and 180 min; cardiac function was assessed 30, 90, 150 and 210 min after beginning the experiment).

Cardiac concentrations of doxorubicinol were determined at various bath concentrations (i.e., 0.9, 1.8, 3.6, 7.2 μM) in atrial strips treated as described above for concentration-response studies. Each strip was removed for assay after a 30 min exposure to a specific doxorubinicol concentration. Cardiac concentrations of doxorubicin and doxorubicinol were determined at 30 min intervals (for 210 min) after adding doxorubicin (175 μM) to atrial strips.

Assay for doxorubicin and doxorubicinol

After removal from the bath, the atrial strip was homogenized with a polytron (Brinkman) for 30 s in 3 ml of iced saline saturated with ammonium sulphate; daunorubicin (500 ng) was then added as an internal standard. The homogenate was extracted with chloroform:isopropanol (5 ml of a 50:50 (v/v) mixture), vortexed for 3 min, and centrifuged at 1000 *g* for 10 min. The organic phase (which contained the anthracycline) was removed and evaporated under N_2 at room temperature. The residue was resuspended in 500 μl methanol and analyzed with a high performance liquid chromatography (h.p.l.c.) system that included a phenyl reversed phase column (4 micron, Radial-Pak, Waters), a programmable infusion pump to control the gradient of the mobile phase, and a fluorescent detector (Kratos; excitation wavelength = 470 nm, emission wavelength = 550 nm). The mobile phase, which initially contained 72% ammonium formate buffer (16 mM; pH 4.0) and 28% acetonitrile (v/v), was changed to 66% ammonium formate and 34% acetonitrile at 6.5 min, and returned to 72% ammonium formate and 28% acetonitrile at 11.5 min. Concentrations of doxorubicin and doxorubicinol were determined from standard curves generated by adding known amounts of doxorubicin and doxorubicinol to atrial tissue treated exactly as above.

Preparation of cardiac SR vesicles

Canine cardiac sarcoplasmic reticulum (SR) vesicles were prepared by a modification of the method of Harigaya & Schwartz (1969). Mongrel dogs of either sex were killed with sodium pentobarbitone. The heart was rapidly removed from the chest and perfused with ice-cold saline. Fat, atrial and right ventricular tissues were discarded. The remaining tissue (i.e., left ventricular free wall and septum) was minced in a food processor (Waring); 40 g of minced tissue was added to 120 ml of buffer (0.9% NaCl, 10 mM Tris maleate, pH 6.8). The buffered tissue was vortexed, homogenized (Brinkman polytron; three 20 s intervals, setting of 4) and centrifuged for 20 min at 4000 *g* at 4°C. Supernatant was collected, filtered through 2 layers of cheesecloth and centrifuged at 8000 *g* for 20 min. The resulting supernatant was centrifuged at 40,000 *g* for 30 min. The pellet (40,000 *g*) was resuspended in buffer (pH 6.8, 0.9% NaCl, 10 mM Tris maleate, 0.3 M sucrose) to achieve a final concentration of approximately 20–25 mg protein ml^{-1}, then stored in liquid N_2. Protein concentrations were determined as described by Lowry *et al.* (1951).

Assay for SR calcium release

The metallochromic indicator, antipyralazo III, was used to measure calcium release according to the technique of Palade & Vergra (1982). Ca^{2+} concentration was determined by subtracting absorbences measured simultaneously at two different wavelengths (absorbence at 710 nm minus absorbence at 790 nm) with an HP 8450A u.v./visible diode array spectrophotometer (Hewlett Packard, Avondale, PA, U.S.A.). Procedures were performed at 32°C; 15 μl (338 μg) cardiac microsomes (22.5 mg protein ml^{-1}) were added to 0.985 ml of a buffered (pH 7.0) solution containing (mM): antipyralazo III 0.3, MOPS (3-[N-morpholino]propanesulphonic acid) 20, KH_2PO_4 50, KCl 5, $MgCl_2$ 2 and ATP 2. Thereafter, calcium chloride (5 nmol Ca^{2+}) was added to load the microsomes with Ca^{2+}; the process was repeated 15 times. Loaded microsomes were then exposed to varying concentrations of doxorubicin or doxorubicinol and rates of calcium release were determined.

To compare effects associated with atrial tissue levels of anthracyclines (doxorubicin or doxorubicinol) with effects of

anthracyclines in isolated subcellular preparations, we converted tissue levels from μg anthracycline per g wet weight to μmol anthracycline per μl of aqueous solution. Because cardiac tissue has a density very similar to that of water (1 g ml^{-1}), a tissue doxorubicinol concentration of 1 μg g^{-1} wet weight is assumed to be equivalent to 1 μg ml^{-1} or 1.8 μM (molecular weight of doxorubicinol is 540).

Materials

Doxorubicin was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Doxorubicinol was synthesized from doxorubicin according to the technique of Takanashi & Bachur (1976). Identity and purity were confirmed by h.p.l.c. as previously described (Olson *et al.*, 1988).

Statistics

One way analysis of variance for unpaired data or randomized block analysis of variance for paired data was used to analyze effects of doxorubicin or doxorubicinol on cardiac mechanical variables as a function of duration of exposure or concentration of anthracycline. Specific contrasts among groups (comparisons at different time points) were made by Duncan's New Multiple Range test. EC_{50}'s (tissue concentation of anthracycline associated with a 50% reduction in measured effect) were compared by probit analysis. Probabilities less than 5% were considered statistically significant ($P<0.05$).

Results

Effect of anthracycline vehicle on cardiac function

Rabbit atria contracting at 2 Hz were stable following addition of 0.9% NaCl to the muscle bath during the 210 min study period (control; Table 1); no substantial changes occurred in any cardiac functional variables at any of the times studied. The largest reductions noted in mean dF/dt were a 6% diminution at 150 min and a 13% decrease at 210 min.

Time-related effects of doxorubicin on cardiac function

Doxorubicin (175 μM) decreased myocardial contractility (dF/dt), impaired cardiac relaxation (90% relaxation time; 90% RT), and increased muscle stiffness (resting force; RF) in a time-related manner (Figures 1 and 2). There was little change in mean dF/dt after a 30 min exposure to doxorubicin (86 ± 11% of pre-doxorubicin value); however, a 90 min exposure decreased dF/dt by more than 50% (48 ± 12% of pre-doxorubicin value), and a 210 min exposure reduced dF/dt by 67 ± 6%. Myocardial relaxation was markedly impaired; 90% RT more than doubled (220 ± 12% of pre-doxorubicin value) during exposure to doxorubicin for 210 min (Figure 2). Resting force, which was not increased after treatment with doxorubicin for 90 min, was significantly elevated at 150 min and further increased at 210 min (136 ± 12% of pre-doxorubicin values) (Figure 2).

Table 1 Changes in dF/dt, RF and 90% RT as a function of time after adding vehicle (0.9% NaCl) to isometrically contracting rabbit atria

Cardiac variable	*Time of exposure to vehicle* 30 min	90 min	150 min	210 min
dF/dt	106 ± 5	101 ± 8	94 ± 10	87 ± 11
RF	101 ± 2	99 ± 2	99 ± 2	100 ± 2
90% RT	102 ± 2	101 ± 3	99 ± 4	98 ± 4

Values are mean ± s.e.mean of 7 experiments and are expressed as percentages of pre-vehicle values. dF/dt, maximum rate of rise of force; RF, resting force-force exerted by atria when not actively contracting (at rest); 90% RT, 90% relaxation time (time for peak developed force to decrease by 90%); for additional information, see Methods.

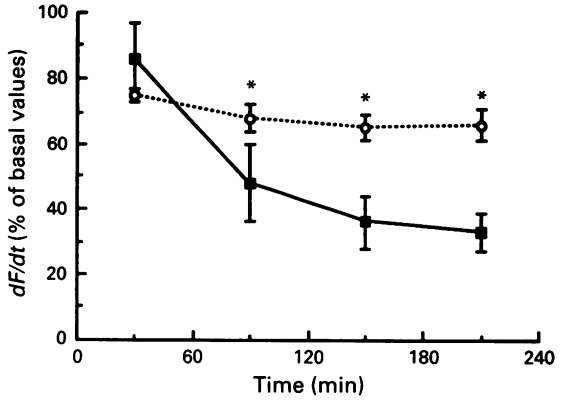

Figure 1 Effects of doxorubicin (Dox ■; 175 μM) and doxorubicinol (Doxol ○; 1.8 μM) on cardiac contractility (dF/dt) as a function of time after adding either agent to isometrically contracting rabbit atria. The x-axis shows duration of treatment with Dox or Doxol; the y-axis shows dF/dt, expressed as percentages of pre-Dox or pre-Doxol values. Data are mean ± s.e.mean ($n = 4$). *$P<0.05$ (for Dox vs Doxol, using 1-way analysis of variance and Duncan's New Multiple Range test).

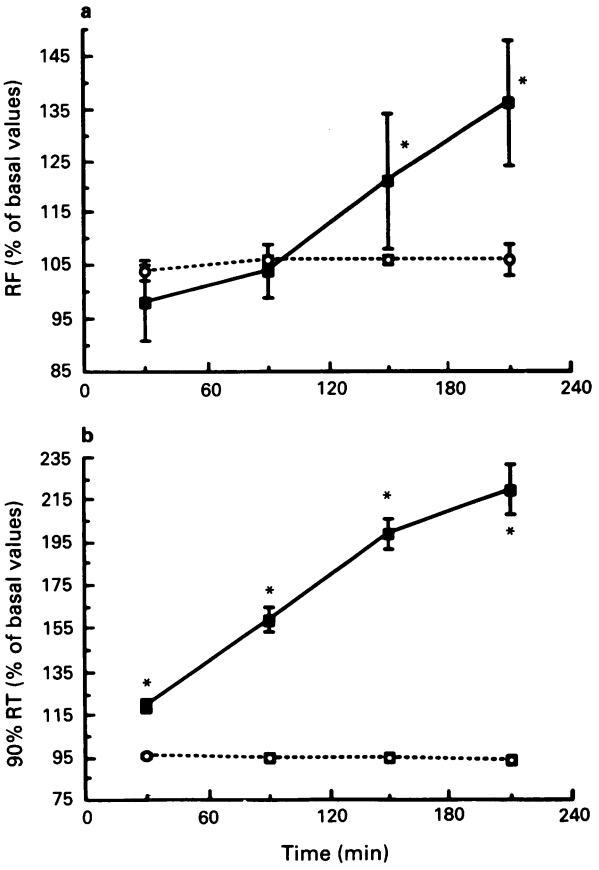

Figure 2 Effects of doxorubicin (Dox ■; 175 μM) and doxorubicinol (Doxol ○; 1.8 μM) on resting force (RF, a) and 90% relaxation time (90% RT, b) as a function of time after adding either agent to isometrically contracting rabbit atria. The x-axis shows duration of treatments; the y-axis shows RF and 90% RT, expressed as percentages of pre-Dox or pre-Doxol values. Data are mean ± s.e.mean ($n = 4$). *$P<0.05$ (for Dox vs Doxol, via 1-way analysis of variance and Duncan's New Multiple Range test).

Table 2 Atrial doxorubicin and doxorubicinol levels following treatment with doxorubicin

	Time (min)						
	30	*60*	*90*	*120*	*150*	*180*	*210*
Dox	70	110	207	183	240	229	239
(s.e.mean, $n = 3$)	26	24	20	24	32	37	34
Doxol	0.09	0.60	0.72	1.57	1.91	3.09	2.62
(s.e.mean, $n = 5$)	0.05	0.04	0.10	0.20	0.35	0.81	0.43
Dox/Doxol	778	183	287	116	126	74	91
% Dox	99.9	99.5	99.7	99.1	99.2	98.7	98.9
% Doxol	0.13	0.54	0.34	0.87	0.79	1.34	1.08
% Dox max	24	40	78	68	98	100	100
(s.e.mean, $n = 3$)	8	6	4	5	8	0	0
% Doxol max	4	18	20	47	57	81	100
(s.e.mean, $n = 5$)	2	3	2	7	11	12	0

Doxorubicin (dox) concentration expressed as μg per g wet weight of tissue, doxorubicinol (doxol) concentration expressed as μg per g wet weight of tissue.
% Dox = % of anthracycline that is Dox (% Dox = Dox/(Dox + Doxol)).
% Doxol = % of anthracycline that is Doxol (% Doxol = Doxol/(Dox + Doxol)).
% max Dox = Dox concentration at each time/highest concentration of Dox during each time course.
% max Doxol = Doxol concentration at each time/highest concentration of Doxol during each time course.

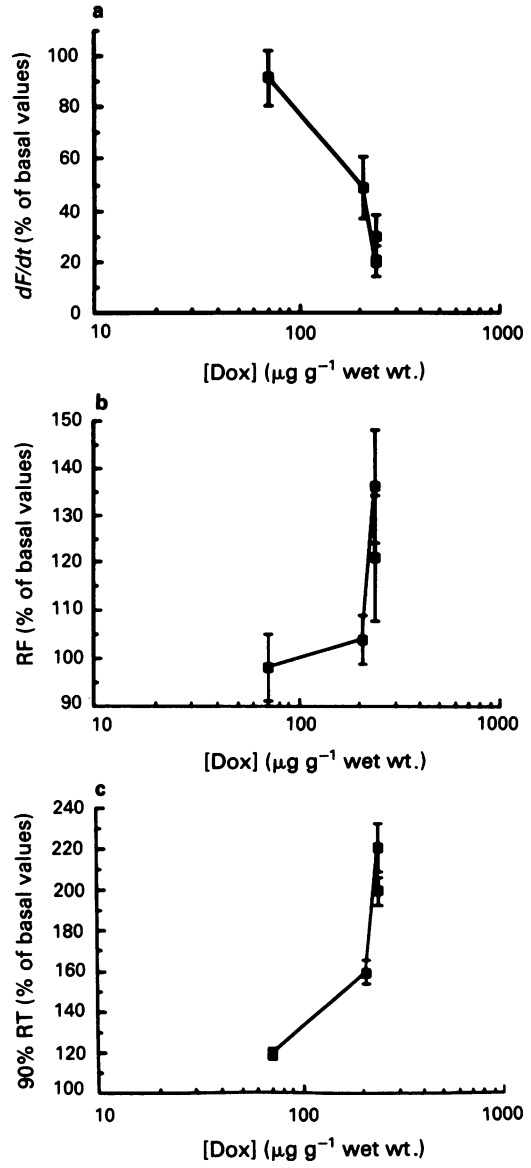

Figure 3 Relationship of atrial doxorubicin (Dox) concentration and changes in cardiac variables (dF/dt, a; resting force, RF, b; 90% relaxation time, 90% RT, c) in rabbit atria treated with 175 μM Dox. The x-axis shows mean ($n = 3$) atrial Dox concentrations; the y-axis shows mean ± s.e.mean ($n = 4$) values of cardiac mechanical variables after 30, 90, 150 and 210 min of treatment with Dox; mechanical variables are expressed as percentages of pre-Dox values.

Time-related effects of doxorubicinol on cardiac function

Unlike doxorubicin, no substantial time-related effects were observed during treatment with doxorubicinol (1.8 μM) for 210 min. Doxorubicinol (1.8 μM) treatment did decrease dF/dt by 25 ± 2% at 30 min and by 32 ± 4% at 90 min, but no further decrease in dF/dt occurred over the next 120 min (i.e., between 90 and 210 min, mean dF/dt decreased by 2%) (Figure 1). Similarly, doxorubicinol significantly increased RF (4 ± 2%; $P < 0.05$) after 30 min, without further increasing RF during the ensuing 180 min (between 30 and 210 min) (Figure 2). In contrast to the ability of doxorubicin to prolong 90% RT, doxorubicinol did not alter 90% RT (Figure 2).

Effects of time on atrial concentrations of doxorubicin and doxorubicinol following exposure to doxorubicin

Treatment of atrial strips with doxorubicin (175 μM) for 210 min produced a time-related increase in atrial concentrations of doxorubicinol (Table 2). Atrial doxorubicin level attained 78% of peak value during the first 90 min of exposure to doxorubicin and increased only 3.4 fold between 30 and 210 min of exposure to doxorubicin (70 to 239 μg g^{-1} wet weight; Table 2). By contrast, doxorubicinol concentration increased 35 fold between 30 and 180 min of exposure (0.088 μg g^{-1} wet weight at 30 min to 3.09 μg g^{-1} wet weight at 180 min), with the most rapid rate of increase occurring between 30 and 90 min (i.e., doxorubicinol level increased more than 8 fold; from 0.088 to 0.715 μg g^{-1} wet weight). During the course of study, the concentration of doxorubicinol increased approximately 10 fold relative to the concentration of doxorubicin ([doxorubicin]/[doxorubicinol] decreased from 778 at 30 min to 74 at 180 min). Nonetheless, on an absolute basis, doxorubicin always predominated over doxorubicinol, never constituting less than 98.7% of the anthracycline (doxorubicin and doxorubicinol) present in cardiac tissue.

Relation between atrial concentrations of doxorubicin and doxorubicinol and changes in cardiac function

Treatment with doxorubicin for 210 min produced alterations of cardiac functional variables (dF/dt, RF, 90% RT) that were directly related to cardiac concentrations of both doxorubicin and doxorubicinol (Figures 3 and 4). The most pronounced effects on functional variables were observed when tissue doxorubicin levels were between 100 and 300 μg g^{-1} wet weight. At a tissue level of 200 μg doxorubicin g^{-1}

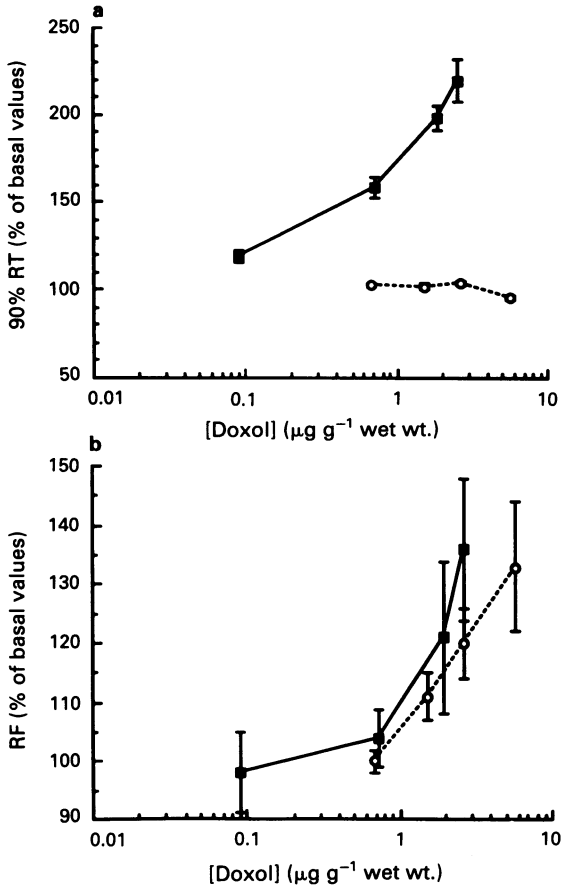

Figure 4 Relationship of diastolic dysfunction and atrial doxorubicinol (Doxol) concentration. Changes in diastolic function (90% relaxation time, 90% RT, a; resting force, RF, b) were evaluated in rabbit isolated atrial strips following exposure to either doxorubicin (Dox; ■; 175 μM) or to doxorubicinol (Doxol; ○). Atrial Doxol concentrations were measured in similarly treated atrial strips. The x-axis shows mean atrial Doxol concentrations (expressed as μg Doxol g^{-1} wet weight) resulting from exposure to Dox for 30, 90, 150 and 210 min (■, $n = 3$) or from treatment with a range of Doxol concentrations (0.9, 1.8, 3.6 and 7.2 μM; ○, $n = 3$); the y-axis shows corresponding mean values of RF and 90% RT, expressed as a percentage of the value immediately prior to addition of Dox ($n = 4$) or Doxol ($n = 7$) to the muscle bath. Data are mean ± s.e.mean.

wet weight, dF/dt had already declined by 50%, whereas RF and 90% RT were just beginning to rise sharply.

Exposure of atria to various concentrations of doxorubicinol (0.5, 1.0, 2.0 and 4.0 μg ml^{-1}) led to increases of doxorubicinol in the atrial strips (0.66 ± 0.10, 1.53 ± 0.20, 2.68 ± 0.52 and 5.72 ± 0.71 μg g^{-1} wet weight, respectively). These data allowed determination of the relation between mechanical dysfunction and concentration of doxorubicinol in heart, without the confounding influence of doxorubicin concentration (Figures 4 and 5). Cardiac doxorubicinol concentration, while unrelated to 90% RT (Figure 4), paralleled the depression of dF/dt (Figure 5) and increase in RF (Figure 4) observed during treatment with doxorubicinol. The functional variable that appeared most sensitive to cardiac doxorubicinol level was dF/dt, which was inhibited by 18 ± 5% ($P < 0.05$) when the atrial doxorubicinol level was 0.66 ± 0.10 μg g^{-1} wet weight (Figure 5). Resting force was not significantly increased until the doxorubicinol level was 2.3 fold higher (1.53 ± 0.20 μg g^{-1} wet weight; RF increased by 11 ± 4%; $P < 0.05$). By contrast, treatment with doxorubicinol never increased 90% RT (Figure 4), not even when atrial doxorubicinol level reached 5.72 ± 0.71 μg g^{-1} wet weight (a level associated with a 54 ± 8% decrease in dF/dt and a 33 ± 11% increase of resting force).

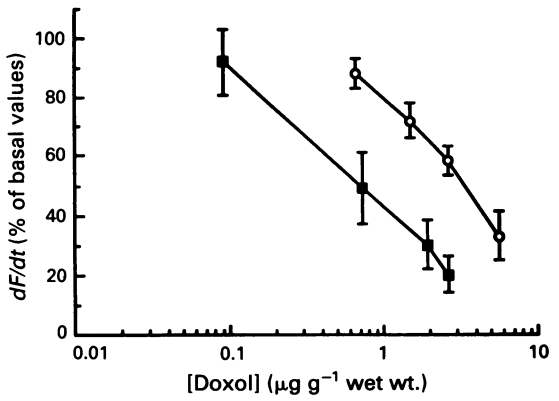

Figure 5 Relationship of contractile dysfunction and atrial doxorubicinol (Doxol) concentration. Changes in contractile function (dF/dt) were measured in isometrically contracting rabbit atrial strips following exposure to either doxorubicin (Dox; ■) or to doxorubicinol (Doxol; ○). Atrial Doxol concentrations were measured in other similarly treated atrial strips. The x-axis shows mean atrial Doxol concentrations (expressed as μg Doxol g^{-1} wet weight) resulting from exposure to Dox (175 μM) for 30, 90, 150 and 210 min (■, $n = 3$) or from treatment with a range of Doxol concentrations (0.9, 1.8, 3.6 and 7.2 μM; ○, $n = 3$); the y-axis shows corresponding mean values of dF/dt, expressed as percentages of values obtained immediately prior to addition of Dox ($n = 4$) or Doxol ($n = 7$) to the muscle bath. Data are mean ± s.e.mean.

The degree of contractile dysfunction associated with any particular concentration of cardiac doxorubicinol depended upon whether the level was elevated by treatment with doxorubicin or treatment with doxorubicinol (Figure 5). The two treatments produced parallel tissue concentration-response relations, allowing for comparisons of EC_{50} (atrial concentration of doxorubicinol associated with a 50% reduction in dF/dt); EC_{50} associated with doxorubicinol treatment was 5 fold greater than the EC_{50} due to doxorubicin treatment (0.7 vs 3.5 μg g^{-1} wet weight).

Effects of doxorubicin and doxorubicinol on SR Ca^{2+} release

Atrial levels of doxorubicin and doxorubicinol resulting from incubation with 175 μM doxorubicin (Table 2) provided the basis for selecting anthracycline concentrations (3 and 10 μM) to study in our experimental model of sarcoplasmic reticulum (SR) calcium release. Doxorubicinol and doxorubicin both caused a concentration-related release of calcium from isolated SR vesicles (Table 3). Doxorubicinol, however, was more potent than doxorubicin, producing a 3 and 15 fold greater stimulation of calcium release at 10 and 3 μM, respectively ($P < 0.01$).

Because anthracyclines can enhance the sensitivity of the Ca^{2+} release channel of SR to Ca^{2+} (increase Ca^{2+} induced-

Table 3 Effects of doxorubicin or doxorubicinol on maximum rates of calcium release from sarcoplasmic reticulum vesicles (SR) from canine cardiac muscle

	Concentration	
Anthracycline	3 μM	10 μM
Doxorubicin	0.8 ± 0.4	59 ± 7
Doxorubicinol	12.0 ± 1.4*	165 ± 29*

Values (nmol Ca^{2+} mg^{-1} protein min^{-1}) are mean ± s.e. mean; $n = 6$ at 3 μM; $n = 4$ at 10 μM. Amount of free Ca^{2+} (nmol) in reaction cuvettes did not differ at the time that the anthracycline was added to release SR Ca^{2+}.
*Indicates significant differences ($P < 0.01$) between doxorubicin and doxorubicinol at each concentration.

Table 4 Doxorubicin (Dox) and doxorubicinol (Doxol) contributions[1] to sarcoplasmic reticulum (SR) dysfunction in Dox-treated atria: estimates based on atrial concentrations of Dox and Doxol and their abilities to cause subcellular dysfunction

	Dox or Doxol contribution to Ca^{2+} release by SR in working atria				
	Potency factor[2]	*Atrial concentration-ratio (Dox/Doxol)* or (Doxol/Dox)‡*		*Percentage contribution*[3]	
Anthracycline	(SR Ca release)	30 min	180 min	30 min	180 min
Dox	0.07	778*	74*	100%	96%
Doxol	15	1/778‡	1/74‡	0%	4%
	Dox or Doxol contributions to inhibition of SR Ca^{2+} ATP-ase in working atria				
	Potency factor[2]	*Atrial concentration-ratio (Dox/Doxol)* or (Doxol/Dox)‡*		*Percentage contribution*[3]	
Anthracycline	(ATP-ase inhibition)	30 min	180 min	30 min	180 min
Dox	0.01	778*	74*	98%	35%
Doxol	100	1/778‡	1/74‡	2%	65%

[1]Assumes direct relationship between cardiac concentration of Dox or Doxol in working atria and effects of these agents on subcellular function.

[2]Potency factors (PF) (ratios of relative potencies to stimulate Ca^{2+} release (data from Table 3) or to inhibit Ca^{2+} ATP-ase (data from Boucek *et al.*, 1987b)); PF_{Dox} = potency of Dox/potency of Doxol; PF_{Doxol} = potency of Doxol/potency of Dox.

[3]Percentage contribution (PC) for Dox or Doxol was computed as follows: $PC_{Dox} = RC_{Dox}/(RC_{Dox} + RC_{Doxol}) \times 100$. $PC_{Doxol} = RC_{Doxol}/(RC_{Doxol} + RC_{Dox}) \times 100$. (RC, in above formula, is defined as the product of potency factor and atrial concentration-ratio. For example, RC for Dox on Ca^{2+} release at 180 min is $0.07 \times 74 = 5$).

Ca^{2+} release; Pessah *et al.*, 1990), it was necessary to control carefully the concentration of free Ca^{2+} during introduction of anthracyclines; no significant differences occurred among groups (nmol free $Ca^{2+} = 1.70 \pm 0.12$, 1.60 ± 0.09, 1.57 ± 0.05 and 1.56 ± 0.07 in doxorubicin 3 μM, doxorubicinol 3 μM, doxorubicin 10 μM and doxorubicinol 10 μM groups, respectively).

Non-anthracyclines that alter Ca^{2+} handling by SR: contractile effects over time

To determine whether non-anthracyclines that affect SR Ca^{2+} availability also cause time-related inhibition of contraction, we added 1 mM caffeine or 20 μM ruthenium red to isolated atria and monitored the cardiac mechanical effects over time (protocol identical to studies with doxorubicin and doxorubicinol). Both caffeine and ruthenium red decreased contractility from pre-drug values by 30 min ($25 \pm 5\%$ and $17 \pm 3\%$, respectively). Neither agent, however, caused further time-related effects on contraction ($13 \pm 6\%$ and $14 \pm 4\%$ decrease in contractility from pre-drug values 210 min after addition of caffeine and ruthenium red, respectively).

Discussion

Doxorubicinol, the C-13 OH metabolite of doxorubicin, was considerably more potent that doxorubicin in depressing mechanical function of rabbit isolated atria. Within the first 30 min of treatment, both doxorubicin (175 μM) and doxorubicinol (1.8 μM) produced a similar degree of contractile depression. However, only doxorubicin caused progressive myocardial dysfunction during the remaining 3 h of the experiment; doxorubicinol failed to produce time-dependent cardiodepressant effects.

Treatment with doxorubicin for 30 min lowered dF/dt by 14%, and lengthened 90% RT by 19%, without changing RF (Figures 1 and 2), whereas a 3.5 h exposure reduced dF/dt by 67%, prolonged 90% RT by 220% and elevated RF by 36%. By contrast, treatment with doxorubicinol (1.8 μM) decreased dF/dt by 25% (no major effect on RF or 90% RT) within 30 min, without further altering cardiac variables during the final 3 h of the experiment (Figures 1 and 2). Thus, despite nearly identical chemical structures (dissimilar only at C-13 moeity—doxorubicin contains a ketone, doxorubicinol an OH group), doxorubicin and doxorubicinol differed markedly in their abilities to produce time-related cardiotoxic effects.

Time-dependent dysfunction could possibly reflect progressive destabilization of *in vitro* preparations. Isolated hearts, for example, may be susceptible to hypoxia or acidosis, owing to ineffective delivery of oxygen or removal of metabolic waste. Such problems, however, would be unlikely in thin strips of muscle (atrial preparations less than 1 mm thick). Also, at 30°C, our muscle preparations are quite stable over time (Table 1); higher temperatures are associated with greater mechanical instability, perhaps owing to imbalances in oxygen supply and demand.

The relationship between time-dependent dysfunction and alteration of cytoplasmic Ca^{2+} needs to be considered. For example, increases in cytoplasmic Ca^{2+} have been reported to provoke myocardial ischaemia (Hearse *et al.*, 1977; Nayler *et al.*, 1979; Carter *et al.*, 1986). However, in our preparation, caffeine (1 mM), which rapidly depressed contractility, failed to produce significant time-related changes, despite its well-known ability to release SR Ca^{2+} (Palade, 1987; Pessah *et al.*, 1987) and increase muscle stiffness (Sutko *et al.*, 1986; Schouten, 1990). Similarly, ruthenium red, a negatively inotropic agent that inhibits Ca^{2+} release from SR (Zimanyi & Pessah, 1991), inhibited contractility within 30 min but failed to depress contractility further over time (Results). Thus, it appears that neither continuous impairment of SR function nor negative inotropic actions alone can account for the time-dependent cardiotoxicity of doxorubicin.

Time-dependency could arise from effects of doxorubicin at multiple subcellular sites (Boucek *et al.*, 1987b; Olson *et al.*, 1981; Floreani & Carpandeo, 1989; Averbuch *et al.*, 1988; Olson & Mushlin, 1990; Gaudiano & Koch, 1991), as a result of differences in time-constants for events mediated by doxorubicin-receptor interactions. Cardiac functional status at any particular time would then reflect a dynamic blending of early, intermediate, and delayed toxic effects of doxorubicin. Influences of anthracyclines on gene expression (selective decrease in synthesis of myofibrillar proteins) have been postulated to contribute to the time-dependent nature of chronic anthracycline cardiotoxicity (Ito *et al.*, 1990), but genetic lesions would seem unlikely to contribute to the pattern of acute cardiotoxicity observed in our preparation.

Our data indicate that doxorubicinol would not be expected to mediate time-related components of doxorubicin cardiotoxicity if such effects were merely caused by SR Ca^{2+} release. Concentrations of doxorubicinol in heart were always quite low, 74 fold–778 fold lower than cardiac concentra-

tions of doxorubicin (Table 2). On this basis, for doxorubicin and doxorubicinol to make equal contributions to dysfunction associated with doxorubicin treatment, doxorubicinol would need to be 74 fold to 778 fold more potent than doxorubicin to impair subcellular functions (Table 4). Our studies with isolated SR, however, indicate that at most, doxorubicinol is 3 to 15 fold more potent than doxorubicin as a stimulator of Ca^{2+} release (Table 3). Therefore, we would not expect the metabolite to disrupt mechanical function via releasing Ca^{2+} from SR, even after prolonged exposure to doxorubicin. Instead, we would predict that doxorubicin, the levels of which in working heart can markedly alter the ability of SR to release Ca^{2+} (Tables 3 and 4), may account for more than 96% of any toxic effect due to enhanced release of SR Ca^{2+} (Table 4).

Doxorubicinol, on the other hand, is an extremely potent inhibitor of various ATPase activities, being nearly 100 times more potent than doxorubicin in inhibiting Ca^{2+}-Mg ATPase and Ca^{2+} loading activities of SR (Boucek *et al.*, 1987b). Such subcellular actions of doxorubicinol (e.g., inhibition of cytoplasmic Ca^{2+} uptake by SR) may lead to mechanical dysfunction (impaired relaxation or diminished contractility) in the working heart, especially as cardiac levels of metabolite increase 8 to 10 fold during prolonged exposure to doxorubicin (Table 2). The analysis in Table 4 suggests that doxorubicinol could be as important as doxorubicin in mediating dysfunction caused by an impairment of Ca^{2+} uptake into SR. Based on this reasoning, we would predict that rising concentrations of C-13 hydroxy metabolite in heart could progressively amplify mechanical effects related to doxorubicin-induced release of SR Ca^{2+}, accounting, at least in part, for the time-dependent component of doxorubicin cardiotoxicity.

Degree of muscle stiffness (RF) was more clearly associated with the cardiac concentration of doxorubicinol than doxorubicin (Figure 4). Indeed, the source of doxorubicinol (whether from treatment with doxorubicin or doxorubicinol) was relatively unimportant as a predictor of the degree of muscle stiffness occurring at any particular concentration of doxorubicinol in heart. The largest increases in RF in doxorubicin-treated preparations, occurred after levels of doxorubicin had peaked and while levels of doxorubicinol were progressively rising. The subcellular basis for the increases in RF is unclear, but our data militate against enhanced release of SR Ca^{2+} as the single, predominant mechanism. Because muscle stiffness reflects the amount of Ca^{2+} at the myofibrillar apparatus during quiescence, agents that interfere with the clearance of Ca^{2+} from cytoplasm can readily influence RF. As mentioned above, doxorubicinol, at least in subcellular systems, inhibits the three primary mechanisms involved in clearing Ca^{2+} from the myofibrils; namely sarcolemmal Ca^{2+} ATPase (Harada *et al.*, 1990), Na/Ca^{2+} exchange (Boucek *et al.*, 1987a) and SR Ca^{2+}-Mg ATPase (Boucek *et al.*, 1987b). Thus, doxorubicinol may decrease cardiac compliance by inhibiting sequestration of Ca^{2+} into SR and/or by interfering with processes that translocate Ca^{2+} from intracellular to extracellular sites.

Impairment of myocardial relaxation (90% RT) appeared to be closely related to the cardiac concentration of doxorubicin but not doxorubicinol (Figure 4). Consistent with this finding, treatment with doxorubicin (175 μM) markedly increased the duration of contraction, whereas doxorubicinol (0.9 μM–7.2 μM) never altered contractile duration (Figure 2). (The highest doxorubicinol concentration that could be used in our study, however, was nearly 25 fold below the doxorubicin concentration because doxorubicinol was a much more potent negative inotrope than doxorubicin.) From a mechanistic viewpoint, impairment of relaxation following treatment with doxorubicin appears to be either (1) independent of the level of doxorubicinol in heart or (2) dependent upon an interaction between doxorubicinol and doxorubicin in heart (Figure 4). In addition, the ability of an anthracycline to impair cardiac relaxation seems to depend more upon its ability to stimulate Ca^{2+} release from SR than upon its propensity to inhibit mechanisms that remove Ca^{2+} from the vincinity of the contractile apparatus (Table 4).

Contractile dysfunction (decreased dF/dt) was directly related to the concentration of doxorubicinol in heart (Figure 5). The degree of contractile depression, however, was highly dependent on the source of cardiac doxorubicinol (Figure 5). The tissue concentration-response curve for doxorubicinol, using data from doxorubicin-treated preparations, was shifted to the left relative to the curve obtained from doxorubicinol treated muscles ($EC_{50} = 0.75$ vs $3.5\ \mu g\ g^{-1}$ wet weight). This concentration-response analysis suggests that doxorubicin and doxorubicinol may interact to depress contractility. Such an interaction may occur as doxorubicin diminishes the SR pool of activator Ca^{2+} by enhancing SR Ca^{2+} release, and doxorubicinol decreases the amount of activator Ca^{2+} through inhibition of cytoplasmic Ca^{2+} uptake into SR.

While the knowledge of tissue concentrations of drugs or metabolites (e.g., doxorubicinol) may provide critical information about pharmacological mechanisms, basing inferences exclusively on tissue levels can be misleading. Such information, for example, tells us nothing about the intracellular location or distribution of doxorubicinol or the concentration of doxorubicinol at putative sites of toxicity (e.g., sarcoplasmic reticulum, sarcolemma, or mitochondria). Cardiac doxorubicinol levels measured after doxorubicinol treatment, could reflect intramyocardial doxorubicinol as well as doxorubicinol adsorbed to nonmyocardial tissue or to outer surfaces of myocytes, whereas cardiac doxorubicinol levels measured after treatment with doxorubicin most likely represent intracellular doxorubicinol, generated by intracardiac anthracycline reductase. As a result, a component of the myocardial dysfunction observed during treatment with doxorubicinol could arise from effects on the exterior surface of the sarcolemma (e.g., ion channels) rather than from (or in addition to) effects at intracellular sites. Further studies will be needed to identify specific, functionally important receptors for doxorubicin and doxorubicinol and to quantify the anthracycline present at such receptors following treatment of cardiac preparations with doxorubicin or doxorubicinol.

In conclusion, our data suggest that the time-related formation of the potent cardiotoxin, doxorubicinol, may contribute to the time-dependency of doxorubicin cardiotoxicity in an isolated, working heart preparation. Owing to its relative abundance in heart, doxorubicin would appear to make a much larger contribution than doxorubicinol to mechanical dysfunction resulting from anthracycline-induced release of Ca^{2+} from SR. On the other hand, doxorubicinol, being considerably more potent that doxorubicin to inhibiting processes that remove Ca^{2+} from the contractile apparatus, could play a role in myocardial dysfunction stemming from impaired clearance of cytoplasmic Ca^{2+}. Thus, our data indicate that time-related increases of doxorubicinol in heart, even in the presence of an unchanging cardiac concentration of doxorubicin, could conceivably amplify the functional impact of doxorubicin-induced release of SR Ca^{2+}. Doxorubicin and doxorubicinol may thereby act in concert to promote cardiac dysfunction *in vitro*.

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