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Daunorubicin Cardiotoxicity

EVIDENCE FOR THE IMPORTANCE OF THE QUINONE MOIETY IN A FREE-RADICAL-INDEPENDENT MECHANISM

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ABSTRACT. Anthracyclines, such as daunorubicin (Daun), and other quinone-containing compounds can stimulate the formation of toxic free radicals. The present study tests the hypothesis that the quinone moiety of Daun, by increasing free-radical production, disrupts sarcoplasmic reticulum (SR) function and thereby inhibits myocardial contractility *in vitro*. We compared Daun with its quinone-deficient analogue, 5-iminodaunorubicin (5-ID), using experimental interventions to produce various contractile states that depend on SR function. At concentrations of Daun or 5-ID that did not alter contractility (dF/dt) of steady-state contractions (1 Hz) in electrically paced atria isolated from adult rabbits, only Daun significantly attenuated the positive inotropic effects on dF/dt of increased rest intervals (PRP; post-rest potentiation) or increased stimulation frequencies. Attenuation was to 98 ± 6% at 1 Hz, and 73 ± 8 and 67 ± 8% for 30 and 60 sec PRP, respectively, and 73 ± 3 and 63 ± 3% at 2 and 3 Hz, respectively, for 88 μM Daun ($P < 0.05$, vs pre-drug baseline values, mean ± SEM). These effects of Daun were similar to those of caffeine (2 mM), an agent well known to deplete cardiac SR calcium. We also examined the effect of Daun in isolated neonatal rabbit atria, which lack mature, functional SR; Daun did not alter the force–frequency relationship or PRP contractions. Additional studies in Ca^{2+}-loaded SR microsomes indicated that both Daun and 5-ID opened Ca^{2+} release channels, with Daun being 20-fold more potent than 5-ID in this respect. Neither anthracycline, however, induced free-radical formation in SR preparations (assayed via nicking of supercoiled DNA) prior to stimulating Ca^{2+} release. Thus, our results indicate that Daun impairs myocardial contractility *in vitro* by selectively interfering with SR function; the quinone moiety of Daun appears to mediate this cardiotoxic effect, acting through a mechanism that does not involve free radicals. BIOCHEM PHARMACOL **60**;10:1435–1444, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. anthracycline; daunorubicin; sarcoplasmic reticulum; ryanodine receptor calcium release channel; free radicals; congestive cardiomyopathy

The anthracyclines Dox†† and Daun are highly effective medications used in the treatment of a variety of cancers. However, they produce a potentially fatal cardiotoxicity that is dependent on the total cumulative dose administered [1]. Despite extensive efforts to understand the pathogenesis of anthracycline cardiotoxicity, the intracellular targets and molecular mechanisms remain elusive.

There is considerable support for the idea that anthracycline cardiotoxicity results from free-radical-induced subcellular injuries. The quinone moiety of anthracyclines can be reduced enzymatically, with subsequent generation of highly reactive oxygen free radicals. These radicals can damage intracellular organelles, such as the SR, disrupting the mechanisms that regulate the SR Ca^{2+} pools essential to the maintenance of normal myocardial contractility [1]. A variety of animal models of anthracycline cardiotoxicity have revealed patterns of cardiac dysfunction that are consistent with anthracycline-induced disruptions of Ca^{2+} homeostasis [1, 2].

The potential link between cardiotoxicity and SR dysfunction has prompted investigators to study the effects of anthracyclines on SR Ca^{2+} homeostasis in both cardiac and skeletal muscle. Dox and Daun have been noted to stimulate the release of Ca^{2+} from isolated cardiac and skeletal muscle SR vesicles as well as from permeabilized ventricular fibers [3–7]. Consistent with isolated SR preparations, anthracyclines increase the probability of open-state conformation of SR Ca^{2+} release channels incorporated into lipid bilayers [8]. Although the initial exposure to

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†† *Abbreviations*: 5-ID, 5-iminodaunorubicin; Daun, daunorubicin; Dox, doxorubicin; DTT, dithiothreitol; PRP, post-rest potentiated; SOD, superoxide dismutase; and SR, sarcoplasmic reticulum.

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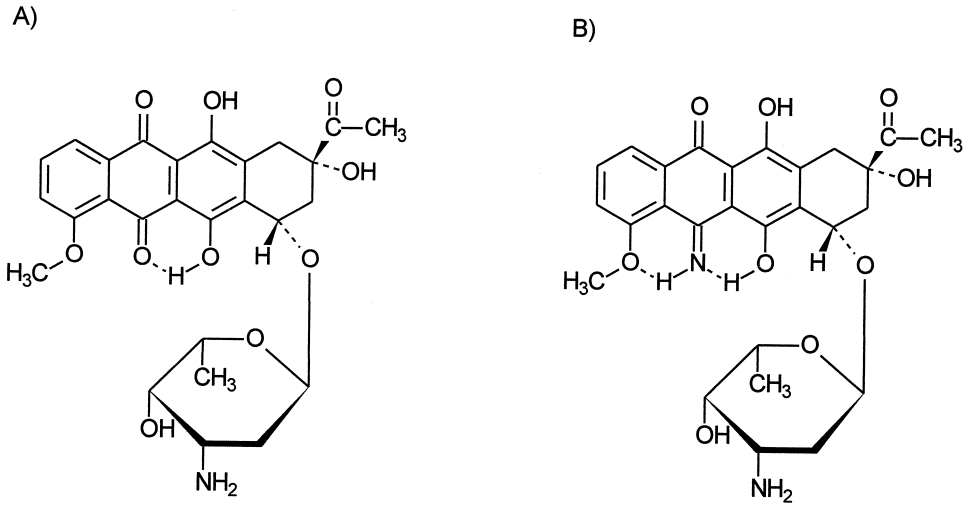

FIG. 1. Chemical structures of the anthracycline antibiotics Daun (A) and 5-ID (B).

doxorubicin opens single SR Ca^{2+} release channels in lipid bilayers, prolonged exposure inactivates these channels [9]. Free radicals have been suggested to mediate the inactivation because pretreatment with DTT prevents the inactivation by anthracyclines [9].

The above studies indicate that anthracycline exposure can disrupt SR Ca^{2+} handling, but the mechanism remains controversial. Some investigators have argued that free-radical processes, dependent on redox cycling of the quinone moiety, are involved. Oxidizing agents, such as metals and hydrogen peroxide, are known to stimulate Ca^{2+} release from skeletal muscle SR by oxidation of Ca^{2+} channel sulfhydryl groups to disulfides [10–14]. However, Feng *et al.* [15] suggest that quinone-containing molecules exert their effects independently of free radicals, directly affecting "hyperreactive" Ca^{2+} channel sulfhydryl groups by producing "local changes in the redox environment."

Despite recent studies demonstrating that anthracyclines induce alterations in SR Ca^{2+} fluxes, it remains unclear how important such effects are to the cardiotoxicity of anthracyclines. Our study was designed to assess the contribution of (i) the quinone moiety to Daun-induced myocardial and SR dysfunction, (ii) SR dysfunction to Daun-induced contractile depression, and (iii) free radical formation to Daun-induced SR Ca^{2+} release. To achieve these aims, we compared the effects of Daun (Fig. 1A) with a quinone-deficient analogue of Daun, 5-ID (Fig. 1B), in a variety of myocardial preparations. Our results provide insights into the molecular basis for the different cardiotoxicity of the two drugs, as well as the importance of the quinone structure to the observed effects.

MATERIALS AND METHODS

Daun and all other chemicals, unless otherwise noted, were obtained from the Sigma Chemical Co. 5-ID was a gift from Farmitalia Carlo Erba. 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.

Muscle Function Studies

Adult (2.5 to 3.5 kg) and newborn (12- to 16-day-old) New Zealand white rabbits of either sex were killed by captive bolt discharge to the cranium (adult) or cervical dislocation (newborn). A median sternotomy was performed immediately, and the heart was removed within 30 sec. After placing the heart in iced Krebs–bicarbonate buffer (*vide infra*), the left atrium was dissected free and divided into two thin strips (adult, 100 mg each); in newborns the whole atrium was utilized. Each preparation was placed in a thermojacketed 25-mL muscle bath (maintained at 30° using a Haake FE/2 circulator) containing Krebs–bicarbonate buffer (127 mM NaCl, 2.5 mM $CaCl_2$, 2.3 mM KCl, 25 mM $NaHCO_3$, 1.3 mM KH_2PO_4, 5.6 mM glucose; the buffer was bubbled continuously with 95% O_2 and 5% CO_2 to maintain pH 7.4). Atrial preparations were fixed to an isometric force transducer (Kulite BG-25) on one end and at the other end to a muscle holder with punctate electrodes at the base. They were stimulated electrically (S88 stimulator, Grass Medical Instruments) by square-wave pulses (3 msec in duration), 10% above threshold voltage. Atrial preparations were stimulated to contract at 1 beat/sec and allowed to stabilize for 90–180 min (when developed force and resting force were demonstrated to be stable and unchanging for 30 min). Cardiac functional variables obtained for each atrial preparation were: maximal rate of rise of force (dF/dt; grams per second) and 90% relaxation time (90% RT; time for peak developed force to decrease by 90%; milliseconds). Variables were obtained using high-speed (100 mm/sec) oscillographic tracings (Gould 4200S oscillographic recorder) and analyzed using a Buxco Pulsatile Analyzer (Buxco Electronics, Inc.). Cardiac variables for each atrial preparation were obtained at contraction frequencies of 1, 2, and 3 Hz (1 Hz = 1 contraction/sec)

and for the first contraction after rest intervals of 20, 30, and 60 sec (PRP20, 30, 60). After obtaining baseline values, Daun or 5-ID was added to achieve cumulative concentrations of 44, 88, 175, or 350 μM in the buffer. Atrial preparations were incubated for 60 min at each cumulative concentration. In separate experiments, the effect on dF/dt of caffeine (2 mM), an agent known to deplete SR Ca^{2+}, was determined at each contraction rate (1, 2, and 3 Hz) and for 20-, 30-, and 60-sec PRP contractions.

Preparation of Cardiac SR Vesicles

Canine cardiac SR vesicles were prepared using a modification of a published method [16] as previously reported [7, 17]. Mongrel dogs of either sex were euthanized with sodium pentobarbital. The heart was rapidly removed from the chest and perfused with ice-cold saline. Fat and 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). Forty grams of minced canine tissue was added to 120 mL of buffer (0.9% NaCl, 10 mM Tris maleate, pH 6.8). The buffered tissue was vortexed, homogenized (Brinkmann Polytron; three 20-sec intervals, setting = 4), and centrifuged for 20 min at 4000 *g* at 4°. Supernatant was collected, filtered through two 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 (0.9% NaCl, 10 mM Tris maleate, 0.3 M sucrose, pH 6.8) to achieve a final concentration of approximately 20–25 mg protein/mL, and then was stored in liquid N_2. Protein concentrations were determined as described by Lowry *et al*. [18].

Calcium Release Studies

A metallochromic indicator, antipyrylazo III, was used to measure Ca^{2+} release by measuring differences in absorbance (710 minus 790 nm) using an HP 8450A UV/Vis diode array spectrophotometer (Hewlett Packard) [19]. Procedures were performed at 32°. Fifteen microliters (338 μg) of cardiac canine microsomes (22.5 mg protein/mL) was added to 0.985 mL of a buffered (pH 7.0) solution containing 0.30 mM antipyrylazo III, 20 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), 50 mM KH_2PO_4, 5 mM KCl, 2 mM $MgCl_2$, and 2 mM ATP. Thereafter, calcium chloride (7 nmol calcium) was added to load the microsomes with calcium; the process was repeated eleven times. Then the loaded microsomes were exposed to various concentrations of Daun or 5-ID, and the rates of calcium release were determined spectrophotometrically.

DNA Cleavage Assays

Plasmid DNA was obtained from New England Biolabs. NADPH cytochrome P450 reductase was a gift from Prof. Steven Aust (Utah State University). All other reagents were obtained from the Sigma Chemical Co. or Fisher Biotech. Enzyme-dependent reactions were performed in a 30-μL volume with 25 mM NaCl, 1 μg pUC19 plasmid, 20 μM EDTA, 10 μM $FeCl_3$, 27 μM drug (Daun or 5-ID), 2 mM NADPH, and 0.0025 U cytochrome P450 reductase. The following reagents were added to some reactions: 6 U catalase, 6 U SOD, 10 mM thiourea. The reaction was incubated at 37° for various amounts of time. Enzyme-independent, SR-dependent reactions were performed in a 200-μL volume containing 1 μg pUC19 plasmid, 0.4 mM ATP, and 0.4 mg/mL of SR protein in a buffered (pH 7.0) solution of 0.30 mM antipyrylazo III, 20 mM MOPS, 50 mM KH_2PO_4, 5 mM KCl, and 2 mM $MgCl_2$. SR reactions were conducted at room temperature for variable amounts of time. Both types of reactions were stopped using phenol/chloroform extraction.

Control experiments using $[Fe(II)\text{-}EDTA]^{2-}$ and H_2O_2 were conducted in a 100-μL volume containing 0.5 μg pUC19 plasmid, 100 μM $[Fe(II)\text{-}EDTA]^{2-}$, and 10 mM H_2O_2. For reactions containing SR, the reaction solution contained the following: 0.4 mM ATP, 0.4 mg/mL of SR protein in a buffered (pH 7.0) solution of 0.30 mM antipyrylazo III, 20 mM MOPS, 50 mM KH_2PO_4, 5 mM KCl, and 2 mM $MgCl_2$. Reactions were stopped after 3 min using 0.8 mM thiourea. Reactions containing SR were phenol/chloroform extracted.

After all of the above reactions, the DNA was ethanol-precipitated, lyophilized, dissolved in 6X dye, and electrophoresed on a 1% agarose gel (1X TAE: 40 mM Tris-acetate, 1 mM EDTA) containing ethidium bromide (7.5×10^{-4} μg/mL) at ~100 V for ~1 hr. Results were visualized and analyzed using an AlphaImager™2000 Documentation and Analysis System. A rectangle was drawn around each band of interest, and the total intensity was integrated.

RESULTS

Cardiac Function

Clinically useful anthracyclines such as Daun may impair cardiac contractility, at least in part, by disrupting SR function [1]. To determine whether the quinone moiety can account for the ability of Daun to both depress contractility and impair SR function, we compared the effects of Daun and 5-ID on contractility (dF/dt) of PRP contractions; the initial contractions following 20-, 30-, and 60-sec rest intervals (rested contractions) were compared with changes in dF/dt at steady state (1 Hz). (Recall that the ability of a rest interval to augment contractility (rested contraction) depends more on SR Ca^{2+} than on Ca^{2+} from other sites [20–24]).

Daun, at 88, 175, and 350 μM, inhibited contractility (dF/dt) of rested contractions more than that of steady-state contractions (Fig. 2A). As the rest interval increased from 20 to 60 sec, Daun caused progressively more inhibition of dF/dt. In contrast, 5-ID did not inhibit dF/dt significantly at any concentration after any rest interval (Fig. 2B).

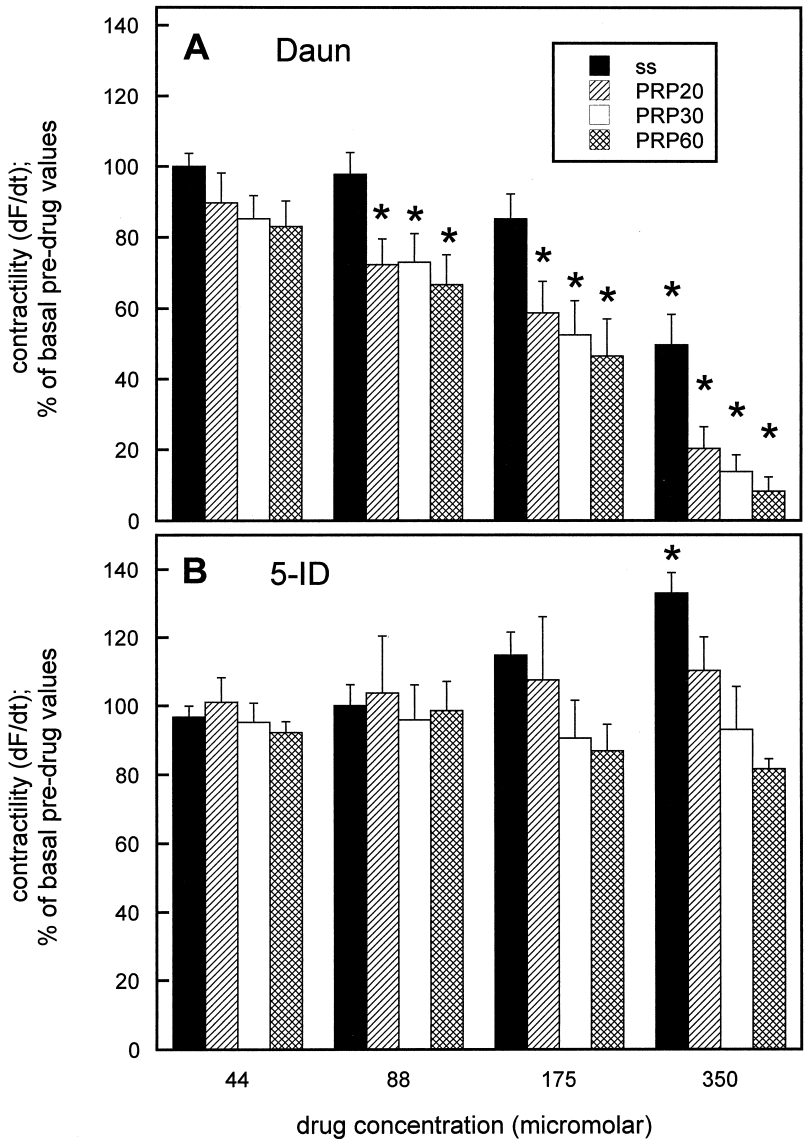

FIG. 2. Contractility (dF/dt; percentage of pre-drug basal values) of PRP contractions as a function of drug concentration for isolated atrial preparations from adult rabbits. Rest intervals were 0 sec (steady-state [ss] contraction at 1 Hz), 20 sec, 30 sec, and 60 sec for Daun (panel A) and 5-ID (panel B). Data are averages of six and four separate experiments for Daun and 5-ID, respectively; error bars show SEM. Key: (*) $P < 0.05$ compared with pre-drug basal values (one-way ANOVA with repeated measures and Duncan's New Multiple Range post-hoc test).

Contractility (dF/dt) at higher rates of contraction also depends more on Ca^{2+} from SR than other Ca^{2+} sites [23, 25]. Daun inhibited dF/dt at 2 and 3 Hz to a greater extent than at steady state (1 Hz) at concentrations of 88, 175, and 350 μM (Fig. 3A). Daun at 44 μM also tended toward inhibition at these contraction rates, but the effect was not significantly different from the control pre-drug values. At 350 μM, exposure to Daun caused inhibition even at steady state (1 Hz). In contrast, 5-ID did not cause a significant inhibition of dF/dt at any concentration or at any rate of contraction (Fig. 3B) and actually increased dF/dt at steady state (1 Hz) at the highest concentration used (350 μM). In addition, as shown in Fig. 4, Daun caused a significant increase in relaxation time compared with 5-ID at concentrations of 88, 175, and 350 μM.

To corroborate the interpretation that selective changes in contractility at high rates of contraction and following rest intervals suggest SR dysfunction, we evaluated the effects of 2 mM caffeine in this experimental paradigm. Caffeine has been used frequently to identify contributions of SR to various pharmacological and physiological processes [20, 22, 25, 26]. Caffeine begins to deplete SR Ca^{2+} stores at concentrations exceeding 1 mM and ultimately can incapacitate myocardial functions that depend upon the availability of SR Ca^{2+}. In addition, caffeine and anthracyclines are known to share the same SR binding site to effect opening of the calcium release channel [4, 5]. Table 1 shows that 2 mM caffeine did not decrease dF/dt of steady-state contractions (1 Hz) but significantly inhibited dF/dt of 20-, 30-, and 60-sec rested contractions and at 2 and 3 Hz. This profile of effects is qualitatively similar to that of Daun, suggesting that the mechanisms of cardiotoxicity may also be similar.

To probe the dependence of Daun cardiotoxicity on SR further, we performed experiments using newborn rabbit myocardium. Newborn rabbit myocardium is less dependent on SR Ca^{2+} transients than is that of the adult [27]. Thus, if the cardiotoxicity of Daun primarily involves

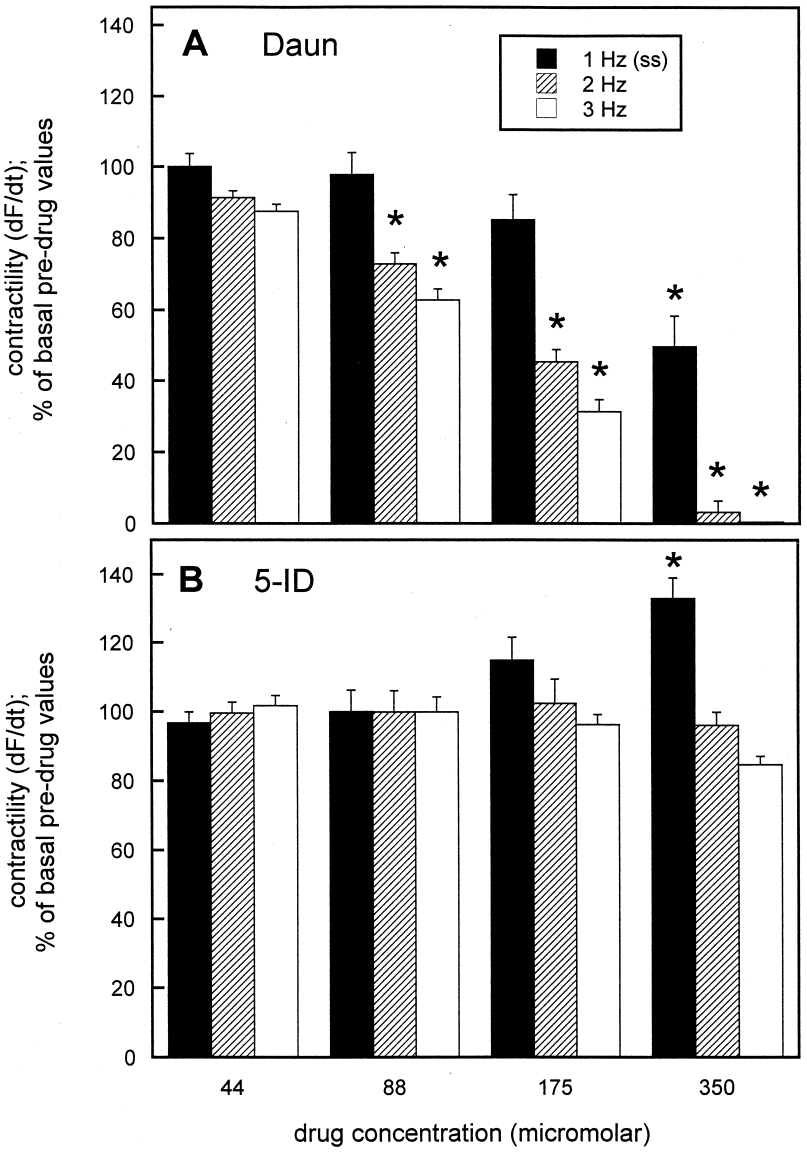

FIG. 3. Contractility (dF/dt; percentage of pre-drug basal values) as a function of buffer drug concentration in isolated atrial preparations from adult rabbits. Values were obtained at contraction rates of 1, 2, and 3 Hz for Daun (panel A) and 5-ID (panel B). Data are averages of six and four separate experiments for Daun and 5-ID, respectively; error bars show SEM. Key: (*) $P < 0.05$ compared with pre-drug basal values (one-way ANOVA with repeated measures and Duncan's New Multiple Range post-hoc test).

dysfunction of SR, we would expect Daun to be less cardiotoxic in newborn than in adult *in vitro* cardiac preparations. Figure 5 shows the effects of Daun (44–350 μM) on dF/dt after 20-, 30-, and 60-sec rested contractions (panel A) and at 1, 2, and 3 Hz (panel B). Daun, at the concentrations used, did not inhibit contractility of rested contractions, nor was there a trend to decrease contractility as the length of the rest interval increased. Daun did produce a frequency-dependent effect on contractility (3 Hz) at the highest concentration used (350 μM), but the decrease in dF/dt was less than that observed in adult preparations.

Ca^{2+} Release Studies

The ability of Daun to selectively decrease contractility (dF/dt) following rest intervals and at high rates of contraction (2 and 3 Hz) and with reduced potency in newborn compared with adult preparations suggests that Daun cardiotoxicity is dependent upon SR Ca^{2+} release. This indicates that cardiotoxicity of Daun, at least *in vitro*, results from perturbation of Ca^{2+} handling by SR and requires the quinone moiety. Thus, we directly tested the effects of Daun and 5-ID on SR Ca^{2+} release, utilizing isolated SR microsomal preparations loaded with Ca^{2+}. Table 2 shows the rates of SR Ca^{2+} release in response to exposure to various concentrations of Daun and 5-ID. Daun caused rapid Ca^{2+} release at all drug concentrations tested (10–300 μM). Ca^{2+} release was initiated within seconds after the addition of Daun and was complete in less than 5 min. In contrast, 5-ID required concentrations in excess of 200 μM to initiate SR Ca^{2+} release. Indeed, to attain a Ca^{2+} release rate comparable to that of Daun, a 20-fold greater concentration of 5-ID was required. Additionally, Daun reached maximum effect at 100 μM, half the concentration of 5-ID required to initiate Ca^{2+} release. Thus, replacement of the quinone moiety with an imino group both decreases cardiac dysfunction (contractility and relaxation) and attenuates SR Ca^{2+} release.

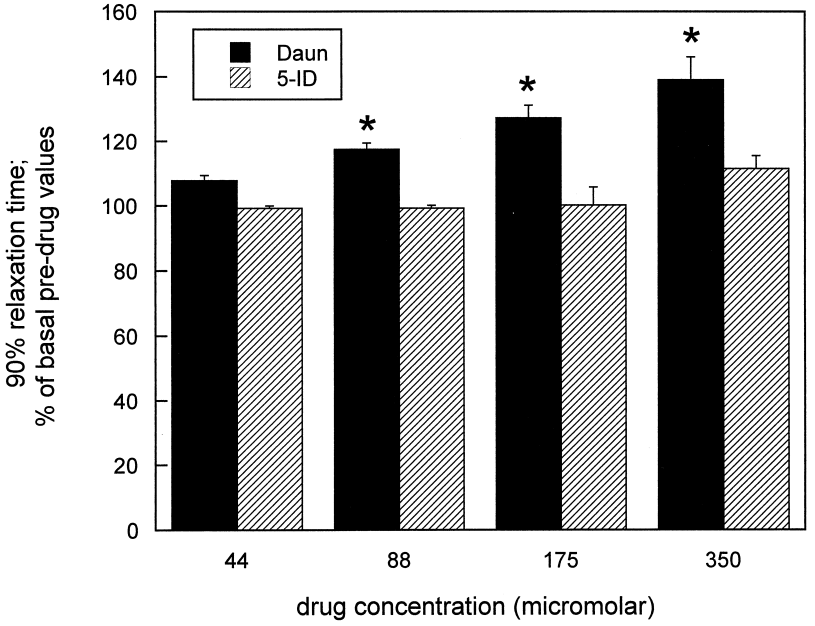

FIG. 4. Ninety-percent relaxation time (percentage of pre-drug basal values) as a function of Daun or 5-ID concentration for isolated atrial preparations from adult rabbits. Values were obtained at 1 Hz. Data are averages of six and four separate experiments for Daun and 5-ID, respectively; error bars show SEM. Key: (*) $P < 0.05$ Daun vs 5-ID at each drug concentration (one-way ANOVA and Duncan's New Multiple Range post-hoc test).

DNA Cleavage Assays

The different Ca^{2+} release rates observed for exposure to Daun versus 5-ID led us to investigate whether this was based on the reported difference in the ability of the two drugs to produce free radicals [28]. Production of free radicals was determined using a plasmid DNA cleavage assay [29, 30]. Using agarose gel electrophoresis, the supercoiled form (form I) of a DNA plasmid can be separated readily from plasmid that, after exposure to free radicals, has undergone single-stranded cleavage (nicked, form II) and double-stranded cleavage (linear, form III).

First, to demonstrate the different abilities of the drugs to cause free-radical damage in this assay system, an enzyme-dependent reaction was performed. Figure 6 shows the results of 60-min reactions in which plasmid DNA was exposed to Daun or 5-ID (30 μM) in the presence of cytochrome P450 reductase and NADPH. The percentage of the total DNA converted from form I to the slower-moving form II reflects the extent of DNA damage. (No form III was observed in this experiment.) Quantitative results are shown in Table 3. Under enzymatic activation, Daun produced more DNA damage than 5-ID. The addition of SOD, catalase, or thiourea (an effective scavenger of hydroxyl radicals) decreased the amount of damage observed for either drug. The enzyme-dependent production of free radicals has been documented extensively for anthracyclines *in vitro* [1]. The reaction mechanism involves the enzyme-dependent transfer of an electron from NADPH to the anthracycline, producing a semiquinone radical. This is followed by electron transfer to molecular oxygen, producing superoxide, followed by production of peroxide and hydroxyl radicals. The results presented here confirm that enzyme-dependent DNA damage involves a free-radical mechanism and that Daun is more potent than 5-ID to produce free radicals in this system.

TABLE 1. Effects of 2 mM caffeine on contractility in isolated adult rabbit atrial preparations

Contraction variable	Contractility (dF/dt) as a percentage of pre-drug basal value*
1 beat/sec (steady state)	129 ± 14
PRP20†	78 ± 5‡
PRP30†	62 ± 6‡
PRP60†	50 ± 2‡
2 beats/sec	81 ± 8‡
3 beats/sec	64 ± 4‡

*Values are means ± SEM, N = 5.

†PRP indicates post-rest potentiated contraction; numerical value indicates rest interval in seconds.

‡Significantly decreased from pre-drug basal value ($P < 0.05$).

The Ca^{2+} release from isolated SR vesicles upon exposure to Daun (or 5-ID) (*vide supra*) is observed in the absence of added cytochrome P450 reductase or NADPH. To investigate the relationship between the Ca^{2+} release and free-radical production, DNA cleavage experiments were conducted under the conditions used to study SR Ca^{2+} release. Very short-duration reactions were used because experimentally observed Ca^{2+} release is completed within several minutes. Figure 7 shows the results of 3- and 5-min reactions in which plasmid DNA was exposed to Daun or 5-ID in the presence of SR. Quantitative results are summarized in Table 4. Neither Daun nor 5-ID caused DNA damage above the level observed for SR alone. This result was independent of drug concentration and independent of time, and was observed even at a Daun concentration (100 μM) shown to maximize Ca^{2+} release.

To ensure that this assay system is capable of detecting free-radical damage at short time periods, several control experiments were conducted. First, DNA was exposed to

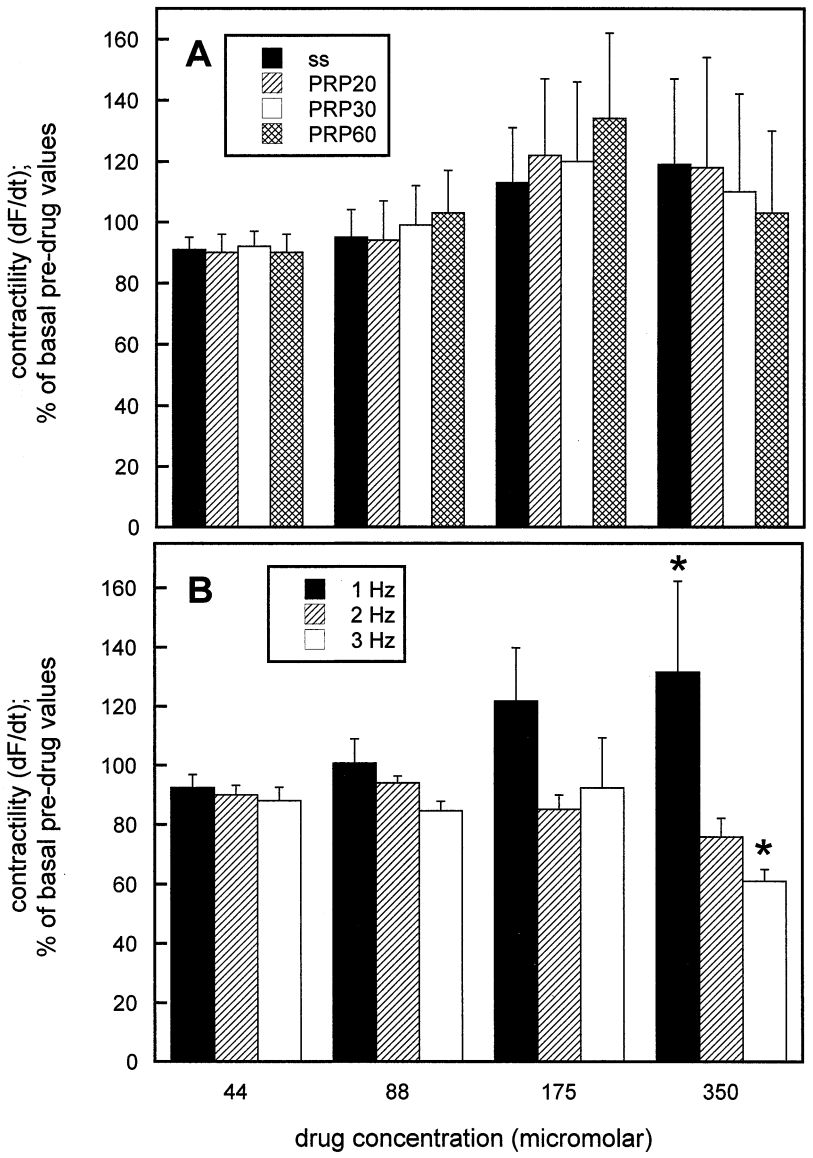

FIG. 5. Contractility (dF/dt; percentage of pre-drug basal values) as a function of Daun concentration for preparations from newborn rabbits. (A) dF/dt for PRP contractions; rest intervals of 0 sec [steady-state (ss) contraction at 1 beat/sec], 20 sec, 30 sec, and 60 sec. (B) dF/dt at contraction rates of 1, 2, and 3 Hz. Data are averages of seven separate experiments; error bars show SEM. Key: (*) $P < 0.05$ compared with pre-drug basal values (one-way ANOVA and Fisher's LSD post-hoc test).

$[Fe(II)\text{-}EDTA]^{2-}$ and H_2O_2 for 3 min. These reagents form hydroxyl radicals via the Fenton reaction [31]. Data are summarized in Table 5 and clearly indicate that free-radical-dependent damage can be visualized over very short time periods using this assay. Second, DNA was exposed to $[Fe(II)\text{-}EDTA]^{2-}$ and H_2O_2 for 3 min in the presence of SR and Daun or 5-ID. Data summarized in Table 5 demonstrate that, while the presence of SR attenuates the free-radical damage to the DNA, the presence of SR and anthracyclines do not prevent visualization of free-radical-dependent damage over these short time periods.

Thus, under the conditions in which SR Ca^{2+} release is observed, neither Daun nor 5-ID caused free-radical damage to DNA. These results indicate that the difference in SR Ca^{2+} release rates of the two drugs cannot be attributed to differences in free-radical production.

TABLE 2. Rate of calcium release from isolated SR as a function of anthracycline concentration

Anthracycline concentration (μM)	Calcium release (nmol Ca^{2+}/mg SR/min)	
	Daun	5-ID
10	21 ± 5*	ND†
30	132 ± 28	ND
100	250 ± 17	ND
125		ND
200		21 ± 2*
300	297 ± 49	

*Values are means ± SEM, N = 3.
†ND = no Ca^{2+} release detected.

DISCUSSION

Our results show that Daun causes selective impairment of contractility (dF/dt) of the initial contraction after a rest interval (Fig. 2A), indicating that Daun perturbs cardiac

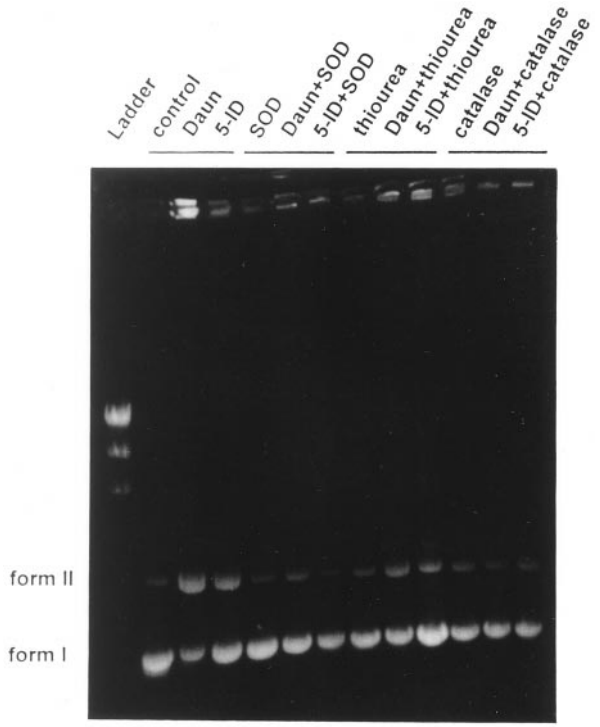

FIG. 6. UV-visualized agarose gel of plasmid DNA cleavage as a function of exposure to Daun or 5-ID (30 μM) in the presence of cytochrome P450 reductase and NADPH (60-min reaction), in the absence or presence of SOD, thiourea, and catalase. Supercoiled DNA is form I, and nicked DNA is form II.

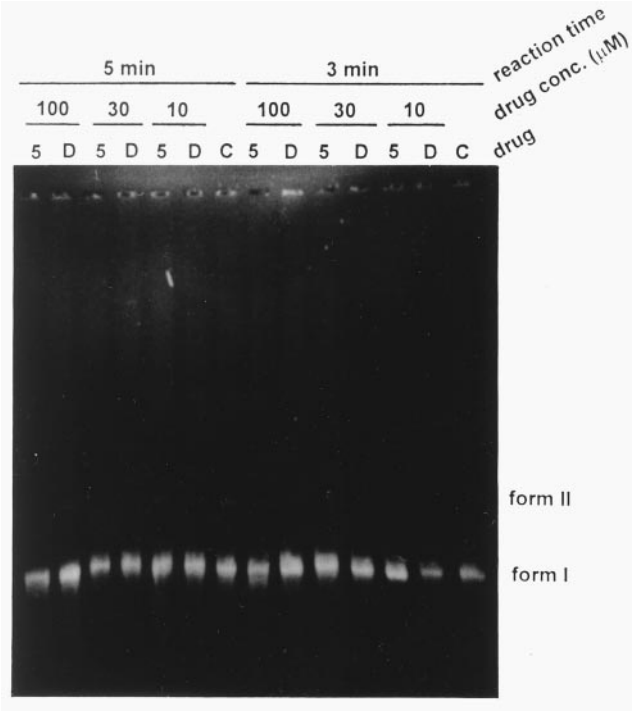

FIG. 7. UV-visualized agarose gel of plasmid DNA cleavage as a function of exposure to SR and Daun or 5-ID under conditions identical to Ca^{2+} release studies. D = Daun; 5 = 5-ID; and C = control (no drug). Supercoiled DNA is form I, and nicked DNA is form II.

function via a mechanism that disrupts cardiac SR function. The first contraction after the rest interval is potentiated because Ca^{2+} from non-releasable SR sites moves to releasable SR sites during the rest. Thus, agents such as caffeine (Table 1) and ryanodine [20], which deplete SR Ca^{2+}, inhibit rested contractions more than steady-state contractions. Conversely, agents such as cobalt and lanthanum, which inhibit transsarcolemmal Ca^{2+} fluxes, inhibit steady-state contractions more than rested contractions [20, 24]. Selective inhibition of contractility at higher rates of contraction, as was observed in this study (Fig. 3A), is also consistent with Daun-induced impairment of SR function because increasing contraction frequency loads the SR with Ca^{2+} [23].

TABLE 3. Enzyme-dependent DNA damage

Reaction conditions*	% Nicked (form II)
Control	21 ± 2†
Daun	49 ± 1‡
5-ID	33 ± 1§
Control + SOD	15
Daun/SOD	20
5-ID/SOD	15
Control + thiourea (TU)	22
Daun/TU	33
5-ID/TU	24
Control + catalase	24
Daun/catalase	21
5-ID/catalase	28

*Drug concentration = 30 μM, reaction time = 60 min; N = 1 unless otherwise noted.
†Mean ± SEM, N = 7.
‡Mean ± SEM, N = 6.
§Mean ± SEM, N = 8.

In contrast to its effects in adult atrial preparations, Daun was relatively ineffective in altering contractility of rested contractions or at high contraction rates of newborn rabbit atrial preparations (Fig. 5). The Ca^{2+} transient that determines contractile function in newborn rabbit myocardium is largely transsarcolemmal; SR becomes a more important source of activator Ca^{2+} as rabbits mature [27]. These observations, taken collectively, suggest that Daun causes cardiac dysfunction *in vitro* by perturbing SR function.

Results show that 5-ID, which has no quinone moiety, is significantly less potent than Daun in effecting changes in cardiac function. This suggests that the quinone moiety is an important structural component of the *in vitro* cardiotoxicity of Daun through a mechanism involving impairment of SR function.

5-ID increased dF/dt at steady state at the highest concentration studied (350 μM). Although the mechanism is unknown, this may be related to other anthracycline-dependent effects. For example, this increase in dF/dt may result from increased sensitivity of the myofilaments to Ca^{2+}, as previously reported for doxorubicin [32].

Daun released Ca^{2+} from calcium-loaded SR prepara-

TABLE 4. DNA damage in the presence of SR and drug

Reaction time (min)	Reaction conditions	Drug concn (μM)	% Nicked* (form II)
3	SR		18 ± 0.6
3	Daun/SR	10	19 ± 2.3
3	Daun/SR	30	18 ± 0.6
3	Daun/SR	100	18 ± 1.9
3	5-ID/SR	10	18 ± 2.3
3	5-ID/SR	30	18 ± 2.3
3	5-ID/SR	100	21 ± 0.3
5	SR		24 ± 1.5
5	Daun/SR	10	22 ± 1.5
5	Daun/SR	30	22 ± 1.3
5	Daun/SR	100	20 ± 2.7
5	5-ID/SR	10	23 ± 0.3
5	5-ID/SR	30	23 ± 1.5
5	5-ID/SR	100	14 ± 1.8

*Values are means ± SEM, N = 3.

tions at concentrations that caused cardiac dysfunction, while 5-ID was approximately 20-fold less active (Table 2). These results are consistent with previous observations for these anthracyclines. Pessah *et al.* [5] reported that 5-ID does not enhance [^{3}H]ryanodine binding to cardiac SR, suggesting that 5-ID does not open Ca^{2+} channels effectively. Similarly, Feng *et al.* [15] demonstrated that 5-ID, unlike quinone-containing compounds, does not decrease the formation of 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) adducts on skeletal muscle Ca^{2+} channel proteins RYR1 and Triadin. CPM is a fluorescent label that forms adducts with reactive sulfhydryl groups. Our results extend the work of Feng *et al.* to show that 5-ID is a poor releaser of Ca^{2+} from cardiac SR. Further, our studies demonstrate a strong relationship between the abilities of anthracyclines to release SR Ca^{2+} and to inhibit contractile function. Thus, SR dysfunction probably accounts for the acute cardiotoxicity of anthracyclines *in vitro* by a mechanism that is highly dependent upon the quinone moiety.

Consistent with previous studies [28, 33], 5-ID was markedly less potent than Daun in producing free radicals in an enzyme-dependent reaction (Fig. 6). However, our results show that under conditions similar to those in isolated SR preparations, both Daun and 5-ID cause SR Ca^{2+} release without generation of free radicals (Fig. 7). These results are consistent with those of Feng *et al.* [15] in suggesting a mechanism for SR Ca^{2+} release by anthracyclines that is free-radical-independent but requires the quinone structure. The functional impairment upon Daun exposure, then, is likely independent of the ability of the quinone to generate free radicals, but may result from direct interaction of the quinone with key components of the SR. Further experiments are required to elucidate the nature of the interaction between the SR and the quinone and the mechanism by which Ca^{2+} channel opening is effected.

How might the acute *in vitro* cardiac effects of Daun and other anthracyclines contribute to the cardiotoxicity that occurs in humans and animals after chronic exposure? The mechanism of chronic cardiotoxicity remains enigmatic. Chronic cardiotoxicity could conceivably result, at least in part, from a summation of acute injuries [2] to organelles, especially in light of the long cardiac residence time of parent anthracyclines and metabolites [34]. Recent data suggest that anthracycline-induced genetic alterations may play an important role in the chronic cardiotoxicity. For example, rabbits receiving multiple doses of Dox over several months exhibit a 50% decrease in the density of cardiac SR Ca^{2+} release channels, as measured by [^{3}H]ryanodine binding [32], and a decrease in gene expression of the SR Ca^{2+} release channel (RYR2) and pump (SERCA2) [35]. Nevertheless, direct effects of anthracyclines on SR Ca^{2+} release channels would only be expected to further compromise SR function already damaged by the effects of anthracyclines on expression of SR proteins.

TABLE 5. $[Fe(II)\text{-}EDTA]^{2-}$ and H_2O_2-dependent DNA damage

Reaction conditions*				
SR	Fe(II) (100 μM)	H_2O_2 (10 mM)	Anthracycline (30 μM)	% Nicked† (form II)
−	−	−	−	11 ± 2.2
−	+	+	−	33 ± 0.5‡§
+	−	−	−	14 ± 0.5‖
+	+	+	−	23 ± 1.0§¶
+	−	−	Daun	16 ± 0.7‖
+	+	+	Daun	22 ± 2.2§
+	−	−	5-ID	12 ± 0.5‖
+	+	+	5-ID	18 ± 0.3§

*Reaction time = 3 min.

†Values are means ± SEM, N = 3 unless otherwise noted.

‡N = 4.

§Indicates result is statistically different from the paired reaction lacking Fe/H_2O_2 ($P < 0.05$, one-way ANOVA, Tukey's test).

‖Indicates result is not statistically different from the control reaction lacking SR/Fe/H_2O_2 (line 1).

¶Result is statistically different from that of the analogous reaction lacking SR (line 2) ($P < 0.05$, one-way ANOVA, Tukey's test).

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