The Major Metabolite of Doxorubicin Is a Potent Inhibitor of Membrane-associated Ion Pumps

A CORRELATIVE STUDY OF CARDIAC MUSCLE WITH ISOLATED MEMBRANE FRACTIONS*

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Doxorubicin (adriamycin) is a highly effective cancer chemotherapeutic drug but its clinical utility is limited by its cardiotoxicity. Doxorubicinol, the major metabolite of doxorubicin, is up to 10 times more potent than doxorubicin at inhibiting isometric contraction of the papillary muscle isolated from the right ventricle of rabbit heart. Doxorubicinol also increases resting tension of isolated cardiac muscle indicative of incomplete relaxation between contractions, a characteristic of doxorubicinol but not of doxorubicin. This study assesses the effect(s) of doxorubicinol on a variety of ion pumps which may explain, in part, the action of the metabolite in the intact muscle.

We find the doxorubicinol is a potent inhibitor (IC50 < 5 μg/ml) of calcium-stimulated ATPase activity of sarcoplasmic reticulum from canine heart and rabbit skeletal muscle. At comparable levels, doxorubicinol is also a potent inhibitor of (Na + K)-ATPase of cardiac sarcolemma and the Mg-dependent ATPase activity referable to the F0F1 proton pump of mitochondria. For each of these ion pumps, doxorubicinol is at least 80 times more potent an inhibitor than doxorubicin. Doxorubicinol, between 10 and 50 μg/ml, increases resting tension up to 4-fold in isolated papillary muscles cyclically contracting at 30 times/min. Resting stress is relatively insensitive to doxorubicin. Thus, doxorubicinol is a potent inhibitor of several key cationic pumps that directly or indirectly regulate cell calcium and inhibits relaxation in the isolated fiber preparation. These observations add a new dimension to understanding the cardiotoxicity of doxorubicin.

EXPERIMENTAL PROCEDURES

Materials—Doxorubicin, A23187, ouabain, AMP, vanadate-free ATP, and antipyrylazo III were obtained from Sigma. [3H]AMP was purchased from New England Nuclear. Doxorubicinol was prepared by chemical reduction of doxorubicin according to the method of Takahashi and Bachur (6). Identity and purity were confirmed by mass spectrometry. The metabolite was collected as a single peak on a reversed phase column (C18) of HPLC. The purity of the metabolite was confirmed by co-chromatography with authentic samples using a high performance liquid chromatography system (7). All stock solutions of doxorubicin and doxorubicinol were prepared in glass distilled deionized water and shielded from light. All pH values given in this text were measured at room temperature unless otherwise stated.

Isolation of Subcellular Fractions—Cardiac muscle sarcoplasmic reticulum (SR) was isolated from adult canine heart as previously described by Chamberlain et al. (8). After isolation, the purified SR was resuspended in 0.29 M sucrose, 0.29 M KCl, and 10 mM imidazole-HCl (pH 6.7) and stored at 70 °C until used. Skeletal muscle SR ("light SR") subfraction referable to longitudinal tubules was isolated from the hind limb muscles of New Zealand White rabbits as previously described by Meissner et al. (9) and modified by Saito et al. (10). Cardiac muscle sarcolemma was isolated from canines using the method previously described by Frank et al. (11). Sarcolemmal membrane vesicles were enriched approximately 25- to 50-fold with respect to both (Na + K)-ATPase and 5-nucleotidase activity from both rabbit and canine heart. After isolation the purified sarcolemma was resuspended and stored at -80 °C until used. The submitochondrial fraction was prepared by differential centrifugation of isolated mitochondria according to the method previously described (12). All buffer solutions contained 10 mM HEPES-KOH (pH 7.0), 100 mM KCl, 10 mM MgCl2, 1 mM EGTA, 0.1 mM vanadate, and 0.2 mg/ml BSA.

The anthracycline doxorubicin (adriamycin) is a highly effective cancer chemotherapeutic drug which is limited by its cardiotoxicity (1, 2). The mechanism(s) of this cumulative dose-dependent cardiotoxicity is unresolved, although evidence is accumulating in support of several diverse mechanisms (1, 2).

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1 The abbreviations used are: SR, sarcoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate.
vesicles were isolated from bovine heart as previously described by Fleischer et al. (12). After isolation, the submicrovesicular chondrials were resuspended in 10 mM HEPES (pH 7.4) containing 0.25 mM sucrose and stored at -80°C until used. The protein concentration was determined according to Lowry et al. (13) using crystalline bovine serum albumin as a standard.

Isolated Papillary Muscles—Right ventricular papillary muscles were isolated and isometric force studied in a muscle bath as previously described (4). Briefly, New Zealand White rabbits of either sex approximately 8 months old and weighing 2.5–3.0 kg were used in all experiments. Water and food were provided ad libitum. Artificial light was provided in 12-h cycles. The rabbit was killed by cervical dislocation and the heart was rapidly removed and rinsed in physiological saline. Right ventricular papillary muscles were isolated and placed in a muscle bath containing Krebs-bicarbonate buffer of the following composition: 127 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl_2, 25 mM NaHCO_3, 5.6 mM glucose, 1.3 mM KH_2PO_4, and 0.5 mM MgSO_4. The bath was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 (pH 7.35–7.40) at 30°C. The base of the muscle was clamped to a pair of punctate platinum electrodes and the tendinous end was affixed to a force transducer (Gould-Statham model UC3). All muscles were electrically stimulated to contract isometrically at a basal frequency of 1.0 Hz, using square-wave pulses (3.0 ms in duration) at a voltage 10% above threshold. Each muscle was gradually stretched to the optimum length for maximum tension development (L_max) at the beginning of each experiment.

Experiments were started when isometric contractile force remained stable for 30 min. Developed and resting forces were taken from high speed oscillographic force traces, converted to tensions (in grams), and averaged for three successive contractions. Resting tension, an index of the extent of fiber relaxation, was defined as the lowest tension achieved between either steady-state or pair-stimulated contractions. Steady-state contractions were defined as stable contractions during repetitive stimulation at a rate of 30/min (0.5 Hz) and at the plateau of the length-tension relationship.

Cumulative dose-tension response experiments were carried out with papillary muscles treated with either doxorubicin or doxorubicinol. Base-line resting tension (p) was determined immediately before the addition of either doxorubicin or doxorubicinol. Additions of either doxorubicin or doxorubicinol were made every 30 min to achieve a cumulative concentration. Before the addition of the subsequent doses, resting tension was recorded first during steady-state contractions and then during contractions with brief (5 contractions) periods of paired stimulation.

At the end of each experiment, the muscle length and wet weight were determined and the cross-sectional area of the cylindrical papillary muscle calculated. Resting stress (gram/mm^2) was calculated from the measured resting tension by normalizing for cross-sectional area calculated from the muscle weight and length as previously described (4). Only data from muscles of cross-sectional areas less than 1-mm^2 were used in this study.

ATPase Activity Assays—Calcium-stimulated ATPase activity in SR-enriched membrane fractions from rabbit skeletal and canine cardiac muscle was determined as described by Chamberlain et al. (8). Activity was determined at 37°C in 20 mM imidazole–HCl buffer (pH 7.4) containing: 100 mM KCl, 4.0 mM MgCl_2, 100 mM sucrose, 30 μM EGTA, 5.0 mM sodium azide, 4 μg/ml A23187, and 0.1 mM ouabain, in the presence of either added calcium (66 μM) or EGTA (1.0 mM). The assay was initiated by addition of ATP (1 mM) and terminated after 5 min by addition of a “stop” solution containing SDS (2%) and EGTA (2.3 mM) and chilling on ice. Inorganic phosphate was determined by the method of Fiske and Subbarow as described by Frank et al. (11). The preparation was rendered permeable prior to the assay by freeze-thaw cycles and exhibited a latency (0) muscle at a varying concentrations of doxorubicinol. The mean control activities for the calcium-dependent ATPase activity for the SR derived from either canine cardiac (A) or rabbit skeletal (B) muscle were 2.47 and 5.97 pmol of Pi/mg.min, respectively. The concentrations of doxorubicinol given in this and subsequent figures is a Dixon plot of the same data with the x intercept indicated as an apparent K_i assuming that the inhibition is noncompetitive.

Mg-stimulated ATPase activity referable to the ATP-driven F_0,F_1 reversible proton pump was determined in submicrovesicular chondrials prepared from bovine heart mitochondria (12) as described by Fleischer and Fleischer (15). The activity was determined at 30°C in 33 mM Tris acetate buffer (pH 7.4) containing: 3.5 mM ATP, in the presence or absence of MgCl_2 (1.7 mM). The assay was initiated by the addition of sample and terminated after 5 min as above by the addition of the SDS/EGTA stop solution. The ATPase activity referable to the mitochondrial ATP-driven proton pump was determined by the difference between phosphate liberated from ATP in the presence and absence of added magnesium ions. This activity was also shown to be azide sensitive in preliminary experiments. 5'-Nucleotidase Assay—5'-Nucleotidase activity, as defined by the rate of phosphate cleavage from [H]AMP, was determined in sarclemma-enriched membrane fractions from canine heart as described (18). Activity was determined at 37°C in 50 mM Tris-HCl (pH 8.5) containing 80 μM MgCl_2 and 20 μM AMP. The assay was initiated by the addition of membrane protein and terminated after 10 min by the addition of 50 mM ZnSO_4 and 50 mM Ba(OH)_2.

Calcium Loading—Calcium loading of membrane vesicles enriched in sarcoplastic reticulum was performed as previously described (17). Briefly, calcium loading was measured with a Hewlett-Packard UV/
**Table I**
The effect of doxorubicin on ion pump ATPase and 5’-nucleotidase activities

<table>
<thead>
<tr>
<th>Membrane-associated enzyme</th>
<th>Source (species)</th>
<th>Number</th>
<th>Control activity (µmol/min for +Doxorubicin (400 µg/ml))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium pump (Ca-ATPase)</td>
<td>Cardiac SR (canine)</td>
<td>3</td>
<td>2.37 ± 0.27, 1.66 ± 0.34, 69.1 ± 7.7%</td>
</tr>
<tr>
<td>Calcium pump (Ca-ATPase)</td>
<td>Skeletal SR (rabbit)</td>
<td>5</td>
<td>5.64 ± 0.78, 5.02 ± 1.1, 89.0 ± 12.6%</td>
</tr>
<tr>
<td>Na/K pump (Na + K-ATPase)</td>
<td>Cardiac sarclemma</td>
<td>4</td>
<td>23.97 ± 5.5, 25.7 ± 3.8, 92.8 ± 5.1%</td>
</tr>
<tr>
<td>FoFl proton pump (Me-ATPase)</td>
<td>Cardiac mitochondria</td>
<td>5</td>
<td>0.38 ± 0.09, 0.40 ± 0.05, 102.1 ± 5.7%</td>
</tr>
<tr>
<td>5’-Nucleotidase (5’-AMPase)</td>
<td>Cardiac sarclemma</td>
<td>3</td>
<td>0.21 ± 0.03, 0.13 ± 0.02, 58.8 ± 1.9%</td>
</tr>
</tbody>
</table>

* Values are the means ± S.E. in: micromoles of P/µg·min for calcium pump and FoFl proton pump, micromoles of P/µg·h for Na/K pump, and micromoles/µg·min for 5’-nucleotidase.

Doxorubicinol is a potent inhibitor of calcium-stimulated ATPase activity of sarcoplasmic reticulum from canine heart and rabbit skeletal muscle (Fig. 2). The doxorubicinol concentration to reach 50% inhibition (IC50) of (Ca + Mg)-ATPase activity is 2.05 µg/ml (3.77 µM) in SR from canine heart and 2.28 µg/ml in SR from rabbit skeletal muscle. The Dixon plot is linear.

Doxorubicinol also inhibits ATP-dependent calcium loading of the same SR subfractions (Fig. 3). The IC50 is 2.68 µg/ml for rabbit skeletal SR vesicles and 4.50 µg/ml for canine cardiac SR, respectively. The Dixon plot is linear (inset).

We did not detect inhibition of the calcium pump of the sarcoplasmic reticulum by doxorubicin at comparable concentrations (not shown). At 100-fold higher concentrations, 400 µg/ml, doxorubicinol inhibited less than 50% of the (Ca + Mg)-ATPase activities of cardiac or skeletal muscle SR preparations (Table I). Thus, doxorubicinol is at least 200 times more potent than doxorubicin in inhibiting the calcium pump of SR.

The Effect of Doxorubicin and Doxorubicinol on Ouabain-sensitive (Na + K)-ATPase of Cardiac Sarcolemmal Vesicles—Doxorubicinol is also a potent inhibitor of canine cardiac sarcolemmal (Na + K)-ATPase. The IC50 of doxorubicinol is 5.40 µg/ml (Fig. 4). The inset shows that the Dixon plot is linear. By contrast, doxorubicin does not appreciably inhibit (Na + K)-ATPase activity at 400 µg/ml (Table I).

The Effect of Doxorubicin and Doxorubicinol on Mg-dependent ATPase in Submitochondrial Vesicles—The Mg-dependent ATPase activity referable to the FoFl proton pump of submitochondrial vesicles is likewise sensitive to inhibition by doxorubicinol. The IC50 is 2.35 µg/ml (Fig. 5). The inset shows that the Dixon plot is linear. By contrast, doxorubicin has no effect on this enzyme at concentrations up to 400 µg/ml (Table I).

The Effect of Doxorubicin and Doxorubicinol on 5’-Nucleotidase Activity of Cardiac Sarcolemmal Vesicles—Doxorubicinol (50 µg/ml) does not inhibit the 5’-nucleotidase activity from sarcolemma of rabbit heart (not shown). Doxorubicin at comparable concentrations has no effect on sarcolemmal 5’-nucleotidase activity whereas at 400 µg/ml, doxorubicinol inhibits 5’-nucleotidase activity approximately 40% (Table I).

The Effect of Doxorubicin and Doxorubicinol on Resting Stress in Papillary Muscles Isolated from Rabbit Heart—Since doxorubicinol inhibits cationic pumps that regulate the resting levels of myoplasmic calcium which plays a major role in regulating resting force (tension or stress), i.e. the extent of myofibrillar relaxation (19), we determined whether doxorubicinol increases resting force. Doxorubicinol (50 µg/ml) increases resting stress, a resting tension parameter normalized for cross-sectional area, 4-fold in papillary muscles cyclically contracting 30 times/min, reaching a stable value approximately 20 min after addition of doxorubicinol (Fig. 6, A and B).
Doxorubicinol Inhibits Membrane Ion Pumps

**FIG. 4.** Effects of doxorubicinol on the sodium-potassium pump of sarcolemma. The (Na + K)-dependent ATPase activity was determined in membrane vesicles enriched in sarcolemma from canine cardiac muscle at varying concentrations of doxorubicinol. The pump activity shown represents the mean of at least two separate determinations performed in duplicate and are expressed as the percent of the activity obtained without addition of doxorubicinol. The mean control activity was 21.4 μmol/mg-h (n = 2).

**FIG. 5.** The effects of doxorubicinol on the FoF₁ proton pump of mitochondria. The Mg-dependent ATPase activity was determined in submitochondrial vesicles prepared from bovine heart. Pump activity shown represents the mean of at least two separate determinations performed in duplicate and are expressed as the percent of the activity obtained without addition of doxorubicinol. The mean control activity was 3.69 pmol/mg-min (n = 2).

C). Further increasing the concentration of doxorubicinol (up to 400 μg/ml) does not significantly increase resting stress (data not shown). Doxorubicin, at cumulatively increasing concentrations up to 400 μg/ml, does not significantly increase resting stress (Fig. 6, B and C). Doxorubicinol also increases the time to 90% relaxation, a parameter referable to the rate of relaxation (272 ± 23 ms versus 349 ± 9 ms; x ± S.E.; p < 0.05; n = 4).

**DISCUSSION**

This study provides new insight into the toxicity of doxorubicin. We find that doxorubicinol, the major metabolite of doxorubicin, is a potent inhibitor of three different types of ion pumps in different subcellular fractions. In this regard, doxorubicinol inhibits the calcium pump from heart and skeletal muscle SR, the Na/K pump of cardiac sarcolemma, and the FoF₁ proton pump of cardiac mitochondria. The IC₅₀ for each ion pump is approximately 5 μg/ml (10 μM) or lower. In this regard, doxorubicinol is similar to quercetin, a flavanoid inhibitor of a broad range of ion pump ATPases including the calcium pump of SR (20). Doxorubicinol, vanadate, and quercetin are the most potent inhibitors reported for the calcium pump of SR.
In sharp contrast, doxorubicin, the parent compound, is not inhibitory to these pumps at comparable concentrations and is partially inhibitory only at 100-fold higher concentration (Table I). In keeping with these findings, previous reports have shown that doxorubicin has little direct effect on a variety of myocardial transporters or enzymes (22-26). However, doxorubicin, in the micromolar concentration range, induces calcium release from skeletal muscle SR (22).7

The resting stress changes due to doxorubicinol (Fig. 6) may be causally related to inhibition of cationic pumps (Fig. 2-5). First, each of the ion pumps inhibited by doxorubicinol in vitro have been implicated as contributing to the regulation of sarcoplasmic calcium, a determinant of resting contractile force (19). The calcium pump of cardiac SR is critical for removal of calcium from the myofilament enabling relaxation (21). The sarcolemmal (Na+/K) pump contributes to regulation of myoplasmic calcium via Na-Ca exchange and the mitochondrial F0F1 proton pump affects the availability of ATP (21). Second, the combination of paired stimulation and doxorubicinol synergistically increases the potency of doxorubicinol to elevate resting stress (not shown), suggesting that the doxorubicinol-induced increase in resting stress is sensitive to the increase in transsarcolemmal calcium influx associated with paired stimulation. Third, the greater potency of doxorubicinol relative to doxorubicin to inhibit cationic pump activities corresponds to the greater potency of doxorubicinol to increase resting stress. Even so, it should be noted that the concentration of doxorubicinol that impairs cardiac relaxation of isolated cardiac muscle (Fig. 6) is about 10-fold greater than the concentration of doxorubicinol that inhibits ion pumps in subcellular fractions prepared from heart (Figs. 2-5). The higher concentration of doxorubicinol required to increase resting stress may simply be due to limitations of diffusion inherent in the superfused model. Additional factors that might reduce the potency in the model include nonspecific protein binding and compensating mechanisms.

Doxorubicin is known to inhibit contraction in isolated cardiac muscle fibers (3, 4), possibly by inducing release of calcium from SR analogous to its effects on isolated skinned fibers and on SR fractions from skeletal muscle (22).7 Doxorubicin, at much higher concentrations (>100-fold), did not increase resting stress during steady-state contraction under the conditions of our study (Fig. 6). Either doxorubicin does not cause calcium release from cardiac SR or it is possible that calcium removal by the calcium pump of the SR and additionally of the plasmalemma may prevent resting calcium levels from building up. New insight provided by this study and relevant to the therapeutic administration of doxorubicin is that the combination of doxorubicin and its metabolite doxorubicinol may act synergistically to affect the function of the SR in cardiac excitation-contraction coupling.

The inhibition of ionic pumps by doxorubicinol is newly described so that the mechanism of its action in this regard has not as yet been studied. The parent compound has been reported to react specifically with cardiolipin in mitochondrial membranes (2). Such interaction with cardiolipin would not explain inhibition of the Na/K pump in the plasmalemma and the Ca2+ pump of sarcoplasmic reticulum since these membranes are devoid of cardiolipin and doxorubicin is 10-fold more potent than the parent compound. Our working hypothesis is that the primary action of doxorubicinol is directly on the transporter protein, since: 1) the inhibitory action is immediate; 2) a linear Dixon plot is obtained for the inhibition suggestive of enzyme-ligand interaction; and 3) the potency of the metabolite is structurally specific. The additional OH group of doxorubicinol is referable to its potent inhibitory action. This hypothesis is not meant to exclude the possibility that reactive free radicals formed by the anthracyclines produce chronic myocellular injury. The chronic toxicity of doxorubicin in animals appears to be related at least in part to the generation of free radicals (1, 2, 31). The metabolite may also be similarly toxic (32).

The implication of these observations is the possibility that metabolic conversion of doxorubicin to doxorubicinol may be a contributing factor in the pathogenesis of the chronic cardiomyopathy associated with the therapeutic use of doxorubicin. Recent experiments suggest that the administration of doxorubicinol over three weeks results in "adriamycin-like toxic syndrome mainly affecting the heart..." (33). We have provided evidence that doxorubicinol is a more potent inhibitor than doxorubicin of three different types of cationic pumps, including the calcium pump of the SR, that regulate intracellular calcium. The inhibition of these pumps may, at least in part, contribute to: intracellular calcium overload (1) and may be related to electron micrographic observations of dilated SR and T-tubules in cardiac muscle fibers following administration of doxorubicin (30); and abnormal cardiac relaxation, which is observed as an early manifestation of the chronic doxorubicin cardiotoxicity both in animal models (28) and in cancer patients (29). We find that the concentration of doxorubicin (400 μg/ml) which impairs cardiac relaxation in the papillary muscle model and inhibits ion pump activities in subcellular fractions is at least 4-fold greater than the concentration measured in plasma after a bolus intravenous injection (7). The IC50 values for doxorubicinol observed in this study are also higher than the levels observed in the plasma following the administration of doxorubicin (7). However, recent evidence suggests that doxorubicinol accumulates preferentially in the myocardium during chronic doxorubicin administration (27, 34). The tissue levels of doxorubicinol correlate with the development of cardiotoxicity (27) and approaches the calculated IC50 values for doxorubicinol observed in this study. The results reported here, in the context of the observation enumerated above, would suggest that the metabolite, doxorubicinol, is a contributing factor to the chronic cardiotoxicity of doxorubicin. The observations reported here add a new dimension to the toxicity of doxorubicin which must be considered with regard to cancer chemotherapy.

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REFERENCES

7 E. M. Ogunbunmi, R. J. Boucek, Jr., and S. Fleischer, manuscript in preparation.
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