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Effect of Low-dose Doxorubicin on Calcium Content and Norepinephrine Response in Rat Aorta

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Abstract—*Doxorubicin (DXR) is a common antineoplastic agent whose clinical utility is limited by development of a dose-related cardiomyopathy. Recent studies demonstrating DXR toxicity in skeletal muscle suggest that this compound may in fact be a general depressant of muscle function. Although previous studies have reported possible indirect actions of DXR on blood vessels, we have investigated the direct effects of this agent on vascular smooth muscle.*

Chronic, low-dose treatment of rats with intraperitoneal DXR (12 mg/kg *total dose over 4 weeks) had no significant effect on body or heart weight, left ventricular water or calcium content, or aortic water or calcium content. Contractile responses to norepinephrine of thoracic aortic strips taken from DXR-treated rats were attenuated by this treatment, and sensitivity* (EC_{50}) *of these strips to norepinephrine was significantly reduced compared to controls. These results suggest that DXR may have physiological effects on vascular smooth muscle function at doses which produce no signs of toxicity in cardiac muscle.*

INTRODUCTION

DOXORUBICIN (DXR) is a common antineoplastic agent which produces a well-characterized cardiomyopathy as its principal side-effect [1]. This cardiotoxicity limits the total dose which can be administered to cancer patients. Laboratory studies aimed at elucidating the mechanism(s) of DXR-induced cardiotoxicity have usually employed short-term, high-dose regimens in a variety of experimental animals to produce a syndrome which mimics the clinically observed cardiac side-effects. However, when clinical toxicity is observed, it is usually the result of chronic accumulation involving several courses of treatment of DXR. Cardiovascular changes, or effects on other organs, have rarely been studied under conditions in which the total cumulative dose of DXR in laboratory animals was both submaximal and noncardiotoxic and was the result of chronic, low-dose drug administration, even though this represents a therapeutic goal in any patient undergoing DXR therapy.

As with any systemically administered drug, DXR is transported to its sites of action via the blood. Thus, the vasculature is exposed to high levels of DXR during a patient's course of treatment. The normal route of DXR administration is intravenous, but the advent of selective intraarterial administration makes knowledge of DXR effects on vascular tissue even more important [2, 3]. Previous reports have suggested several possible actions of DXR on vascular smooth muscle, but few studies have directly examined the question of DXR's vascular effects. Since the myotoxic effects of DXR are not limited to cardiac muscle, but have recently been demonstrated in skeletal muscles as well [4], we felt a further investigation into possible DXR effects in smooth muscle was warranted. In addition, we consider it important to look for vascular alterations that might occur under conditions of chronic, noncardiotoxic dosing.

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METHODS

A. *Experimental protocol*

Male Sprague–Dawley rats (370–500 g) were allowed access to food and water *ad libitum*. Seven experimental animals were given 1 mg DXR + 5 mg lactose/kg body wt/day, while controls ($n = 5$) were given 6 mg lactose/kg body wt/day. (These doses derive from the manufacturer's clinical compounding of DXR in a 1:5 ratio with lactose.) All solutions were made in 0.9% saline and administered intraperitoneally for 3 consecutive days, fol-

lowed by a 4 day resting period. Dosing was continued for 4 consecutive weeks, yielding a total cumulative DXR dose of 12 mg/kg. This schedule has been shown to minimize mortality and produce no anatomic or physiologic evidence of cardiomyopathy or congestive heart failure in rats [5]. Body weights were recorded daily.

Following the course of treatment, animals were stunned and decapitated. The heart and thoracic and abdominal aortae were quickly removed. The heart was separated into left and right halves, rinsed with saline, blotted and weighed. The left ventricle was then excised and weighed separately. All cardiac tissues were placed in separate vials and frozen for later drying and analysis. The thoracic aorta was cut into proximal and distal halves. The distal half, along with the abdominal aorta, was flushed with saline, cleaned of fat and connective tissue, blotted and weighed. These samples were also placed into separate vials and frozen for later drying and analysis.

B. *Dose–response curves*

The proximal half of the thoracic aorta was carefully cleaned of fat and connective tissue and cut into a helical strip [6], beginning with the distal end and cutting at approximately a 75° angle [7]. Strips were mounted isometrically under 1 g tension in 100 ml double-jacketed water baths containing a Krebs–bicarbonate solution of the following composition (mM): NaCl, 119.7; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 18; glucose, 10; EDTA, 0.026; ascorbic acid, 0.11. pH was maintained at 7.3–7.5 by continuous aeration with 95% O_2–5% CO_2, while temperature was held at 37 ± 1°C. Strips were equilibrated for at least 1 h; bath fluid was changed every 15 min during the equilibration. After equilibration, cumulative dose–response curves were obtained by sequential addition of increasing concentrations of norepinephrine. The total volume of norepinephrine added did not exceed 5% of the bath volume. Responses were recorded through Narco F-60 myographs connected to a Narco Mark IV physiograph. At the end of each experiment, strips were dried to constant weight. The maximum contraction of each strip was expressed as a function of the dry weight of the strip, while EC_{50} values were determined from the dose–response curves and defined as that concentration of norepinephrine which produced one-half maximal contraction.

C. *Tissue analysis*

Heart, abdominal aortic and distal thoracic aortic samples were dried to constant weight at 100°C in acid-washed porcelain crucibles. Thoracic aortic and left ventricular water contents were determined as the difference between wet and dry weights and expressed as a percentage of wet weight. Total dry heart weight was similarly determined.

Left ventricular and abdominal aortic samples were prepared for calcium analysis by wet ashing [8]. 0.5 ml of 18 M HNO_3 was added to each tissue sample. The mixture was heated at 100°C for 1 h in a drying oven until all tissue was dissolved and the solution was clear. Aortic samples were then diluted to 2.0 ml and ventricular samples to 5.0 ml with 0.1% HNO_3. Analyses were performed on an Instrumentation Laboratory Model 251 atomic absorption spectrophotometer. Absorption responses were linear over the concentration range used. Standard curves were prepared using appropriate dilutions of $CaCl_2$ in 0.1% HNO_3.

D. *Chemicals*

Norepinephrine and ascorbic acid were obtained from Sigma Chemical Co. DXR was generously donated by Dr. J.J. Ragolia, Jr. (Adria Labs, Columbus, Ohio). All other chemicals were of analytical grade and obtained from Fisher Chemical Co.

E. *Data analysis*

All values are expressed as mean ± S.E.M. Statistical comparisons were made using Student's t-test with a significance level of $P < 0.05$.

RESULTS

At the end of 4 weeks DXR-treated rats averaged a 6% weight loss while control rats gained 6% of their initial body weights. However, average body weights for control and DXR-treated animals at the end of the 4-week treatment period did not differ significantly from their respective initial body weights.

Table 1 summarizes the cardiac and aortic tissue analyses from control and DXR-treated rats. None of the comparisons (control vs. DXR-treated) in the table demonstrate statistical significance.

DXR treatment caused a shift of almost one order of magnitude in the norepinephrine EC_{50} values obtained on the thoracic aorta as compared to values from control animals (Table 2). Maximum contraction values were reduced by DXR treatment, but comparison with controls did not reach the level of statistical significance.

DISCUSSION

The objective of our study was to test the effects of chronic, low-dose, non-toxic DXR treatment on vascular smooth muscle responses. The maximum tolerated cumulative clinical dose of DXR is 550 mg/m^2, as irreversible cardiotoxic effects become much more prominent above this level [1]. The total cumulative dose of DXR in our experiments was 12 mg/kg administered over a 4-week interval. Using equations derived by Freireich

Table 1. *Comparison of body weights and muscle analyses from control and DXR-treated rats*

	Control (n = 5)	DXR (n = 7)
Body weight (g)		
Initial	422 ± 17	443 ± 14
4-Week	447 ± 17	415 ± 9
Heart weight (g)	0.24 ± 0.01	0.24 ± 0.01
Heart weight/body weight (%)	0.054 ± 0.001	0.057 ± 0.002
Left ventricular water content (%)	76.9 ± 0.4	77.0 ± 0.9
Aortic water content (%)	12.3 ± 5.4	12.2 ± 1.2
Left ventricular calcium content (mg/g dry wt)	0.18 ± 0.01	0.16 ± 0.02
Aortic calcium content (mg/g dry wt)	0.67 ± 0.05	0.71 ± 0.14

Table 2. *Norepinephrine response in aortae from control and DXR-treated rats*

	Control (n = 5)	ADR (n = 7)
Maximum contraction (mg/mg dry wt)	798 ± 180	565 ± 126
EC_{50} ($M \times 10^{-8}$)	0.43 ± 0.03	2.35 ± 0.46*

*$P < 0.01$ vs. control.

et al. [9], this is equivalent to a total dose of about 100 mg/m^2 (95.7–105.0), well below the equivalent therapeutic limit.

Toxic doses of DXR in experimental animals cause severe weight loss or reduced weight gain [10], myocardial calcium accumulation [10–12], and an increase in ventricular water content [11, 13], in addition to a number of cardiac structural abnormalities visible at both the light and electron microscopic level [11, 14]. The present study minimized the possible production of cardiotoxic effects in several ways. First, we chose a dosage schedule which allowed intermittent, chronic administration of the drug over several weeks rather than the acute administration of an equal amount of DXR. This dosage schedule has previously been shown to cause body weight loss but have no significant effect on heart weight and cause no overt signs of cardiac failure in rats [5]. A somewhat similar study in mice investigated the distribution of DXR and toxicity-related deaths using single intravenous doses of DXR compared with an equivalent amount administered in four smaller daily doses [15]. Peak DXR concentrations in both heart and serum and toxic deaths were all significantly lower in mice treated with close repeated doses when compared to those treated with single bolus injections. Both the total dose and the time over which it is administered have been shown to influence the development of DXR-induced cardiotoxicity in clinical studies as well [16, 17].

We administered DXR by the intraperitoneal route to minimize the bolus accumulation of DXR at its sites of action in the body that can occur during normal intravenous administration. Although not common, the intraperitoneal route has been used to effectively treat ovarian carcinoma while at the same time keeping plasma DXR levels low due to a decreased systemic absorption from the peritoneal fluid [18].

While our DXR-treated animals did experience a slight weight loss, it was much less than that which occurs during acute or chronic administration of cardiotoxic doses [10]. Further evidence for the lack of DXR-induced toxicity can be seen in Table 1. Left ventricular, as well as aortic, water content and calcium concentrations were not changed by DXR administration, nor was heart weight or heart weight as a function of total body weight different from that in lactose-treated control animals. Since ventricular calcium accumulation has been shown to precede the development of DXR-induced cardiomyopathy [11, 14], our data indicate that the dose schedule used in the present study did not cause cardiotoxicity. Thus, our DXR-treated animals were not cardiotoxic by any of the above criteria; in addition, they appeared healthy and active, consuming near normal amounts of food and water.

The literature on the vascular effects of DXR contains conflicting reports, most of them presenting data derived from indirect assessments of vascular function. Several studies have noted no vascular effects or injury to vascular tissue caused by DXR [4, 18–20]. Indeed, it has been stated that 'vascular lesions have not been described within the therapeutic dose range' [19], in spite of a previous report

that suggested 'Adriamycin may produce a primary insult to the vasculature' [11]. Vascular insults, of course, may be either morphologic or physiologic. However, others have suggested direct vasoconstrictor [21–23] and vasodilatory [24] effects of this drug. Still another report suggests that DXR may act indirectly on vascular smooth muscle by releasing endogeneous histamine or catecholamines, the effects of which then lead to ischemia that ultimately results in the typical DXR cardiomyopathy [25]. In preliminary experiments using isolated rat aortic strips, DXR in concentrations up to 9.2×10^{-3} M occasionally caused a slight vasoconstriction when added to the bathing medium (Dalske, unpublished observations). This concentration of DXR is more than ten times greater than that which has been shown to abolish contraction in isolated chick hearts [26], depress respiration in isolated chick heart mitochondria [26] and produce several deleterious electrophysiological effects on isolated dog [27] and rat [28] ventricular muscle.

Since direct vascular actions of DXR on isolated rat aorta appear minimal, we also investigated the effects of DXR on norepinephrine-induced vascular smooth muscle contraction. DXR had a striking, depressant effect on norepinephrine responses in aortae from treated animals. The sensitivity of these preparations to norepinephrine was more than five times less than that of control aortae, even at a total dose of DXR which had no apparent cardiotoxic effect. Concurrent with this effect on sensitivity to norepinephrine was a small reduction in the maximum contractile force developed in DXR-treated aortae. Tritton and Yee [29] have demonstrated the cytotoxicity of DXR when it has been rendered incapable of entering cells, thus suggesting that the cell membrane may be a primary site of action for this compound. DXR has also been shown to have a number of effects on cell membrane components, including hormone receptors [30]. Recently, Robison and Giri noted a decrease in agonist binding to cardiac beta receptors in rats treated chronically with DXR [31]. It is not unreasonable to postulate that similar interactions might occur with adrenergic receptors on vascular smooth muscle cells.

In summary, we have demonstrated that chronic low-dose treatment with DXR can induce changes in vascular smooth muscle responses to norepinephrine. It is important to note that these changes, unlike DXR-induced alterations in cardiac muscle function, were not preceeded by any change in vascular calcium or water content. The total dose administered to our experimental animals was equivalent to less than one-fifth the maximum tolerated dose in man and well below the accumulated dose that produces cardiotoxicity.

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