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Absence of Acute Doxorubicin-Induced Dysfunction of Heart Mitochondrial Oxidative Phosphorylation and Creatine Kinase Activities (42699)

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Abstract. Since reductions in cardiac high-energy phosphate content and dysfunction of mitochondrial activities have been demonstrated after doxorubicin exposure, one mechanism of doxorubicin cardiotoxicity has been thought to be an interference with mitochondrial energy metabolism. To determine whether mitochondrial dysfunction is induced by acute drug exposure, isolated rat hearts were perfused with 10^{-5} *M* doxorubicin for 70 min followed by mitochondrial isolation. Rates of electron transport, creatine kinase activity, acceptor control, respiratory control, and ADP/O ratios were assayed and correlated to doxorubicin-induced abnormalities in left ventricular function. At doses of doxorubicin sufficient to cause a marked deterioration of left ventricular systolic pressure and a rise in end-diastolic pressure, no decreases were noted in the measured mitochondrial parameters with either glutamate plus malate or succinate as respiratory substrates. In fact, in some cases the rates of electron transport were higher in mitochondria isolated from the treated hearts. In addition, isolated heart mitochondria were directly incubated in doxorubicin at doses as high as 10^{-4} *M* for up to 70 min at 0 and 20°C and 1.5 min at 37°C. Under these conditions functional impairment of mitochondrial respiration was also not detected. Therefore, it appears that acute doxorubicin cardiotoxicity cannot be related to primary mitochondrial defects in high-energy phosphate metabolism. These data lend further support to the notion that doxorubicin cardiotoxicity may be fundamentally related to changes in coronary vascular resistance and resultant damage induced by hypoperfusion.

In humans, chronic doxorubicin administration produces a dose-dependent cardiomyopathy with clinical congestive heart failure, which limits the utility of this effective anti-cancer agent (1–4). In patients who develop doxorubicin cardiotoxicity, there is abnormal left ventricular function, as documented by invasive (4) and noninvasive (5) diagnostic testing. Chronic administration of doxorubicin to animals also produces a dose-dependent cardiomyopathy (6, 7) which has greatly facilitated the study of this problem.

Acute alterations in cardiac function have also been demonstrated to result from doxorubicin exposure in humans (8) and in animal preparations (9–13). Thus, chronic ventricular dysfunction may result from myocardial damage associated with repeated episodes of acute doxorubicin cardiotoxicity in the setting of chronic drug administration. While chronic cardiomyopathy develops after a total cumulative dose of 500 mg/m^2 body surface area, considerable individual variability exists. A significant study compared two dosing regimens—one group of patients received once weekly doxorubicin and a second group received one-third the individual dose three times per week. Thus, both groups had the same total cumulative dose, but doxorubicin cardiomyopathy developed much earlier in the group receiving the higher individual dose once weekly (14). This study suggests the importance of acute events related to peak drug levels in the development of chronic cardiomyopathy. Therefore, studies of acute cardiotoxicity induced by doxorubicin are important for the further understanding of chronic doxorubicin cardiomyopathy and, in fact, may also provide a rational basis for the development of cardioprotective measures.

During acute doxorubicin exposure, alterations in cardiac intracellular high-energy

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phosphate metabolism have been detected utilizing phosphorus-31 nuclear magnetic resonance (12, 15, 16). Since abnormal mitochondrial structure has characteristically been observed in the setting of chronic drug administration in humans (4, 17) and animals (6, 7, 17, 18), it has been suggested that interference with mitochondrial function may be of central importance in the etiology of doxorubicin cardiotoxicity. Studies with isolated cardiac mitochondria have demonstrated doxorubicin binding to cardiolipin in the inner mitochondrial membrane, which can alter the binding of such enzymes as cytochrome *c* oxidase (19) and creatine kinase (20, 21). Respiration of isolated cardiac mitochondria has been measured after incubation with doxorubicin and has been found to be inhibited usually at doxorubicin concentrations greater than 10^{-5} *M* (22, 23), although one study demonstrated reduction in oxidative phosphorylation at much lower doses (24).

Alternatively, some investigations have found alterations in nuclear structure after doxorubicin exposure with minimal or no changes in mitochondrial structure (10, 11, 17, 19). These data raise the possibility that mitochondrial dysfunction may be an epiphenomenon. Previous investigations have not correlated myocardial performance with mitochondrial dysfunction. Therefore, the present study was undertaken to examine the potential role of mitochondrial dysfunction in acute doxorubicin-induced cardiotoxicity. The data suggest that at doxorubicin doses which clearly induce ventricular dysfunction and reductions in intracellular high-energy phosphates (12), there are no detectable reductions in mitochondrial oxidative phosphorylation or creatine kinase activities. The data are discussed from the view that the mechanism of acute doxorubicin cardiotoxicity must be the result of something other than a primary defect in mitochondrial high-energy phosphate metabolism.

Materials and Methods. *Isolated perfused hearts.* Male Sprague–Dawley rats, 4 months old, were heparinized and anesthetized with pentobarbital. Their hearts were quickly removed and placed in chilled perfusate prior to perfusion by a modified Langendorff method. The ascending aorta was cannulated and perfused with a solution containing 140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 4 m*M* Hepes, and 10 m*M* glucose. The pH was adjusted at 37°C to 7.40 with NaOH. In experimental hearts the perfusate also contained doxorubicin 6 mg/liter (1.03×10^{-5} *M*). The pH of the perfusate was measured after addition of doxorubicin and was also found to be 7.40. The perfusate was bubbled continuously with 100% oxygen. Perfusate flow was 15 ml/min delivered by a peristaltic pump which maintained a constant flow rate at any level of coronary resistance. A stable heart rate of 225 beats/min was achieved via right atrial pacing. The left ventricle was vented and a thin-walled rubber balloon, tied to the end of a fluid-filled length of PE 190 tubing, was inserted into the left ventricle. The cannula was connected to a pressure transducer by way of a 3-way stopcock which allowed constant recording of ventricular systolic and diastolic pressures. Preload was increased by increasing the intraventricular balloon volume.

After aortic cannulation and perfusion, the intraventricular balloon volume was adjusted to produce near maximal developed pressure and this volume was held constant for the duration of the experiment. Hearts were then randomly assigned to a control group ($n = 13$) or doxorubicin group ($n = 12$). After a brief stabilization of the preparation and the acquisition of baseline measurements of end-diastolic pressure and developed pressure, additional determinations of these parameters were made at 10-min intervals during 70 min of perfusion with doxorubicin (experimental group) or under identical conditions without doxorubicin (control group). At the end of 70 min all hearts were removed from the perfusion apparatus for mitochondrial isolation.

Mitochondrial isolation. Hearts were placed in a sucrose-mannitol solution, pH 7.2, at 4°C and finely minced (25, 26). After trypsin digestion and homogenization, cellular debris were separated by differential centrifugation at 483*g* for 10 min at 2°C. The supernatant solution containing the mitochondrial fraction was decanted carefully. The mitochondrial fraction was then isolated by three further centrifugations at 7735*g* and washings. The final mitochondrial pellet was

resuspended in 50 μl sucrose–mannitol. Protein determination was performed by the biuret method (27). The final protein concentration was between 40 and 60 mg mitochondrial protein/ml.

Incubation of isolated heart mitochondria with doxorubicin. Theoretically, the toxic effect of doxorubicin on cardiac mitochondria could be reduced by interaction of the drug with nonmitochondrial intracellular substances. To control for this possibility, mitochondria were isolated from groups of four normal, nonperfused hearts obtained after heparinization and decapitation of 4-month-old Sprague–Dawley rats. The isolated mitochondria were then incubated for varying periods of time with 10^{-5} and 10^{-4} *M* doxorubicin at 2, 20, or 37°C. After incubation, mitochondrial function was determined as described below.

Oxygraph determinations. Clark oxygen electrode studies were conducted separately at 37°C for each mitochondrial fraction in a 3.2-ml chamber (28). The oxygraph medium contained 130 m*M* KCl, 2 m*M* KH_2PO_4, 1 m*M* $MgCl_2$, 10 m*M* glutamate plus 10 m*M* malate, or 10 m*M* succinate (with 5 μ*M* rotenone) as respiratory substrate, 0.5 m*M* K_2-EGTA [ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid], and 5 m*M* Hepes. The pH was adjusted to 7.20 at 37°C. The rates of resting and stimulated oxygen uptake were measured three times for each mitochondrial fraction and the values were averaged. Endogenous rates (State 2), maximum ADP-stimulation rates (State 3), and recovery rates (State 4) of oxygen consumption were measured after the pulsed addition of 640 nmole of ADP. Acceptor control and respiratory control ratios as well as ADP/O ratios were calculated from these polarographic data.

Creatine kinase assay. Assay of the mitochondrial creatine kinase reaction was performed by measurement of the maximum rate of ATP production by the reverse reaction,

phosphocreatine + ADP →

creatine + ATP,

at 30°C, pH 7.40 using a standard hexokinase, glucose-6-phosphate dehydrogenase regenerating system to measure the spectrophotometric rates of NADPH production (28). To assure that the hexokinase-glucose-6-phosphate dehydrogenase system was not rate limiting, the rate of NADPH production was measured in the presence of 150 μ*M* ATP and found to be more rapid than when formed by the reverse creatine kinase reaction.

Statistical methods. Data analyses were performed on a Clinfo PDP 11/60 computer system utilizing unpaired *t* tests to compare measured parameters of mitochondrial function between groups. Repeated measures analysis of variance, performed on a Data General MV 8000 computer, was used to compare serial measurements of ventricular function in different groups of hearts. Data are presented as means ± SEM.

Results. *Isolated perfused hearts: Left ventricular function.* The initial baseline developed pressure (systolic minus end-diastolic pressure) in the 12 experimental hearts, prior to doxorubicin exposure, was 88.8 ± 3.3 mm Hg. In the control group of 13 hearts the initial developed pressure was 79.8 ± 4.5 mm Hg (P = NS). In doxorubicin-exposed hearts, developed pressure rose to a peak level of 105.7 ± 5.6% of the initial baseline pressure after 40 min, and thereafter decreased to 88.4 ± 6.4% of baseline by 70 min. The developed pressure in control hearts slightly but steadily decreased to 90.3 ± 6.0% of baseline ($P < 0.02$, experimental vs control).

The end-diastolic pressures were similar in control and experimental hearts before doxorubicin perfusion: 8.8 ± 1.4 mm Hg in experimental hearts and 10.4 ± 0.7 mm Hg in the control group (P = NS). Changes in end-diastolic pressure during doxorubicin and control perfusion are shown in Fig. 1. In control hearts the end-diastolic pressure remained relatively constant and was 100.2 ± 5.4% of baseline at 70 min. During doxorubicin perfusion, however, the end-diastolic pressure rose markedly to 627.6 ± 150.8% of baseline pressure after 70 min ($P < 0.0001$, experimental vs control). These alterations in left ventricular function are typical of the responses induced by acute doxorubicin exposure (12).

Respiratory parameters in isolated perfused heart mitochondria. In both doxorubicin-exposed and control hearts the mito-

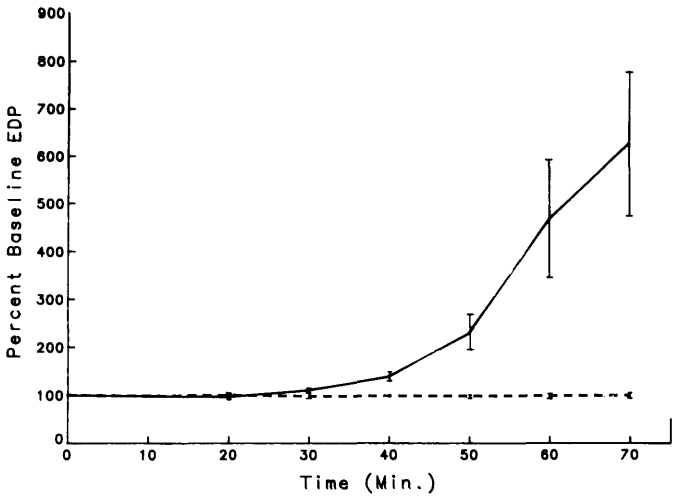

FIG. 1. End-diastolic pressure (EDP) in 12 doxorubicin-perfused (1.03×10^{-5} *M*) (solid line) and 13 control hearts (dashed line) during 70 min perfusion shown as percentage baseline EDP. Standard error bars are included.

chondria isolated after perfusion appeared to be well coupled, since the respiratory transitions occurred with appropriate sharpness. Respiratory parameters of the isolated mitochondria were determined with both electron transport chain Complex I (glutamate + malate) and Complex II (succinate) substrates. The latter series of studies was performed because it has been reported that the reactions of Complex II are quite sensitive to inhibition by doxorubicin treatment (23). Table I presents the data when mitochondria are respiring on a combination of glutamate and malate as respiratory substrate. State 2, the endogenous rate observed prior to the addition of ADP, was slightly higher in mitochondria isolated from doxorubicin-exposed hearts than in mitochondria from control hearts. Interestingly, State 3 rates of respiration were markedly higher in experimental mitochondria as compared to control mitochondria. State 4 respiration, the post-ADP rate, was also slightly higher in experimental than in control hearts. However, the acceptor control, respiratory control, and ADP/O ratios were not significantly different in the two groups (Table I).

When Complex I is inhibited by rotenone and the mitochondria are respiring with succinate as the substrate (Table II), there were only slight differences in any of the respiratory parameters. Clearly, a marked decrease in State 3 was not observed.

Mitochondrial creatine kinase activities. Since a reduction in intracellular high-energy phosphate levels could result from reduction in the activity of mitochondrial creatine kinase, the activity of this enzyme was assayed. The specific activity of mitochondrial creatine kinase was not reduced in mitochondria isolated from hearts exposed to doxorubicin. The creatine kinase activity was 2.49 $\pm$ 0.16 IU/mg mitochondrial protein in experimental hearts and 2.70 $\pm$ 0.11 in control mitochondria (P = NS).

Isolated perfused hearts selected for maximum functional damage. The reduction in left ventricular pressure development caused by doxorubicin perfusion varied from heart to heart. Since the experimental group included all hearts studied, the mean reduction in developed pressure caused by doxorubicin administration was not remarkably different from that observed in the control group of hearts. It was possible, therefore, that a doxorubicin-induced reduction in mitochondrial function in the experimental group was not observed because the final systolic functional differences were minimal. In order to determine whether there was, in fact, a reduction in mitochondrial function in those hearts having a major decrease in left ventricular

TABLE I. MITOCHONDRIA ISOLATED AFTER HEART PERFUSION: ALL HEARTS GLUTAMATE PLUS MALATE AS THE RESPIRATORY SUBSTRATES

Mitochondrial function	Control	Doxorubicin	*P*
Number of hearts	13	12	
State 2	50.5 ± 2.0	61.8 ± 2.1	0.0008
State 3	287.9 ± 29.5	414.3 ± 43.2	0.02
State 4	66.8 ± 3.1	88.2 ± 5.8	0.004
Acceptor control ratio	5.6 ± 0.5	6.5 ± 0.6	NS
Respiratory control ratio	4.3 ± 0.4	4.7 ± 0.4	NS
ADP/O ratio	2.4 ± 0.1	2.3 ± 0.1	NS

systolic function, six experimental and six control hearts were analyzed separately. These hearts were selected from the hearts described above according to the following criteria:

A. Initial baseline mean developed pressure in the experimental and control groups was not significantly different;

B. reduction in left ventricular developed pressure in the experimental hearts to less than 80% of the initial baseline pressure after 70 min perfusion with doxorubicin; and

C. in control hearts there was a minimal reduction in left ventricular developed pressure (final developed pressure greater than 80% of the baseline pressure).

In these selected groups the initial developed pressure in the experimental hearts was 97.2 ± 2.4 mm Hg. In the control hearts the initial developed pressure was 89.8 ± 3.6 mm Hg (*P* = NS). The developed pressures in the two groups of selected hearts are shown in Fig. 2. In control hearts developed pressure remained stable and was 98.3 ± 7.7% of baseline after 70 min. In doxorubicin-exposed hearts the final developed pressure was reduced to 71.3 ± 2.0% of baseline ($P < 0.02$). The marked effect of doxorubicin on the end-diastolic pressure was also observed in these selected hearts and was similar to that shown in Fig. 1.

Separate analysis of the mitochondrial function of these selected hearts was performed. Although the experimental group demonstrated a large reduction in left ventricular developed pressure and the control group had stable systolic function, there was no measurable reduction in mitochondrial function observed in doxorubicin-perfused hearts. In fact, the opposite occurred. The rates of States 2, 3, and 4 respiration, using glutamate and malate, were actually increased in the doxorubicin preparations as compared to the control groups. The data are similar to those presented in Table I. The ADP/O ratios were similar in mitochondrial fractions from the two groups of hearts with a small reduction in creatine kinase activity in the experimental hearts (2.17 ± 0.27) as compared to controls (2.94 ± 0.07; $P = 0.02$). Although the reduction in creatine kinase activity was statistically significant, the magnitude of this reduction was small when compared to the magnitude of left ventricular functional changes.

TABLE II. MITOCHONDRIA ISOLATED AFTER HEART PERFUSION: SUCCINATE AS THE RESPIRATORY SUBSTRATE

Mitochondrial function	Control	Doxorubicin	*P*
Number of hearts	6	6	
State 2	221.9 ± 14.8	247.3 ± 18.0	NS
State 3	789.3 ± 26.3	777.6 ± 66.2	NS
State 4	294.1 ± 20.1	310.1 ± 20.1	NS
Acceptor control ratio	3.5 ± 0.2	3.3 ± 0.3	NS
Respiratory control ratio	2.7 ± 0.1	2.6 ± 0.3	NS
ADP/O ratio	1.8 ± 0.1	1.7 ± 0.1	NS

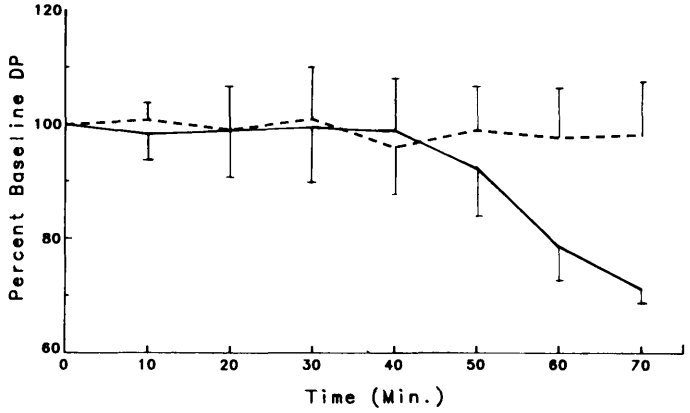

FIG. 2. Developed pressure (DP) in six selected doxorubicin-perfused (1.03×10^{-5} *M*) (solid line) and six selected control hearts (dashed line). In these selected hearts there was a marked reduction in DP in the doxorubicin-exposed group.

Effects of doxorubicin on isolated mitochondria. To determine whether the absence of doxorubicin-induced mitochondrial dysfunction described above was the result of an intracellular nonmitochondrial protective factor which reduced mitochondrial damage, the direct effects of doxorubicin incubation on isolated mitochondria were studied. Mitochondria isolated from normal rat hearts were incubated with 10^{-5} and 10^{-4} *M* doxorubicin for 70 min at either 2 or 20°C. Results from a more stringent third condition were also determined, with 1.5 min incubation at 37°C. Mitochondrial function was evaluated and compared to control mitochondria incubated under identical conditions but without doxorubicin. Incubation for 70 min at 37°C was also conducted, but mitochondrial function deteriorated completely under these conditions in both the control and doxorubicin groups, probably as a result of lysosomal contamination of the mitochondrial fraction. At all concentrations of doxorubicin, all durations of incubation, and all temperatures, there were no differences in the parameters of oxidative phosphorylation when compared to controls. Typical results are shown in Table III, for mitochondria incubated for 70 min with either 10^{-5} or 10^{-4} *M* doxorubicin at 20°C. These results were not substrate specific, since succinate respiration in doxorubicin-treated mitochondria was similar to that in the control group.

The direct effects of doxorubicin on the specific activity of mitochondrial creatine kinase were also assayed in normal isolated mitochondria. No reduction in the activity of this enzyme was observed after incubation of mitochondria with either 10^{-5} or 10^{-4} *M* doxorubicin for 5 min at 37°C. In control mitochondria (eight determinations) the activity of creatine kinase was 4.78 ± 0.18 IU/mg mitochondrial protein. After incubation with 10^{-5} *M* doxorubicin (six determinations) the creatine activity was 4.79 ± 0.17 ($P = 0.96$). Mitochondrial creatine kinase activity was 4.86 ± 0.10 after incubation with 10^{-4} *M* doxorubicin (six determinations; $P = 0.71$).

Discussion. During contraction the isolated, perfused rat heart consumes high-energy phosphate (ATP) at a remarkable rate, approximately 1 μmole per second per gram wet weight of tissue (30). Since the ATP content of the heart is approximately 5 μmole per gram (30), this means that in the absence of regeneration, ATP would be totally depleted in less than 5 sec of contraction. Another way to view this is that the total ATP pool is recycled 12 times per minute. Phosphocreatine, which is present in the myocardial cell at a content of about 10 μmole per gram tissue, adds only an additional 10 sec of energy reserve. Thus, the total pool of the high-energy phosphates turns over four times per minute (30). Since more than 90% of the ATP produced by metabolism is generated

TABLE III. INCUBATION OF NORMAL MITOCHONDRIA, 70 MIN, 20°C: GLUTAMATE PLUS MALATE AS THE RESPIRATORY SUBSTRATES

Mitochondrial function	Control	10^{-5} *M* Doxo	*P* vs Control	10^{-4} *M* Doxo	*P* vs Control
Number of runs	4	4		4	
State 2	103.5 ± 14.5	117.0 ± 21.1	NS	102.0 ± 13.6	NS
State 3	388.0 ± 51.5	325.0 ± 58.7	NS	386.0 ± 58.5	NS
State 4	142.0 ± 17.4	136.8 ± 16.9	NS	142.0 ± 15.4	NS
Acceptor control	3.8 ± 0.2	2.9 ± 0.3	0.04	3.9 ± 0.3	NS
Respiratory control	2.7 ± 0.1	2.4 ± 0.2	NS	2.7 ± 0.2	NS
ADP/O	2.1 ± 0.1	2.0 ± 0.1	NS	2.3 ± 0.1	NS

Note. Respiratory rates are expressed in nanogram atoms O/min/mg mitochondrial protein.

by the Kreb's cycle and oxidative phosphorylation (31), it is quite logical that the adriamycin-induced decline in the high-energy phosphates might be due to a basic defect in mitochondrial metabolism caused by the drug. Even if such a defect were minimal, at such high rates of turnover the heart might eventually progress into a supply/demand imbalance reflected by a decline in the high-energy phosphates.

In these experiments isolated perfused rat hearts developed reduced left ventricular systolic and increased diastolic pressures when coronary perfusate contained 10^{-5} *M* doxorubicin. In our previous studies (12), hearts perfused under these conditions had similar abnormalities in left ventricular function which were associated with a doubling of coronary vascular resistance and a reduction in intracellular high-energy phosphate content (ATP and phosphocreatine). The current study extends these observations by directly comparing the function of mitochondria isolated from doxorubicin-perfused hearts with mitochondria from control hearts. Control hearts had stable left ventricular function and have also previously been shown by ^{31}P NMR spectroscopy to have stable levels of intracellular high-energy phosphates and coronary resistance (12). Despite these functional differences, we observed no reduction in the parameters of oxidative phosphorylation or creatine kinase activity in mitochondria isolated from the doxorubicin-perfused hearts. Because control and doxorubicin-exposed mitochondria were studied under identical conditions, an intrinsic doxorubicin-induced defect is excluded, but alterations in the intracellular environment in experimental hearts, such as decreased pH or decreased substrate delivery, could have altered mitochondrial function.

Since there is inherent biological variability in the responses to doxorubicin, we observed quite different degrees of reduction in systolic function. Because of this the mean reduction in developed pressure was not significantly greater in the doxorubicin-perfused hearts than in control hearts when all hearts were considered. To determine whether this might have masked a reduction in mitochondrial function in those hearts which did show the greatest toxicity (largest decrease in developed pressure), we specifically analyzed the mitochondrial function in six separately selected experimental and six selected control hearts. In these hearts the initial developed pressures were similar with a marked reduction in developed pressure in the doxorubicin-exposed hearts as compared with the stable function seen in control hearts. In this setting important abnormalities in mitochondrial function, if causative of reduced ventricular function, should have been most amplified. Despite this selection (for the "worst case"), mitochondrial function of the doxorubicin-perfused group was still similar to that of controls. There was, however, a slight reduction in creatine kinase activity with doxorubicin exposure.

Commonly used dosages of doxorubicin in human pharmacologic studies are in the range of 30 to 60 mg/m^2 body surface area for each acute administration. Previous studies have measured a triphasic pattern of drug disappearance from plasma (32–35). Using the calculation [concentration × time] to estimate drug exposure, a 60 mg/m^2 dose re-

sults in a 2.6 μM-hr exposure (35). In the present study rat hearts were perfused with 10^{-5} M doxorubicin for 70 min. This corresponds to a drug exposure of 11 μM-hr which, while high, is within a factor of 4 of that measured in humans.

Since previous studies have documented reduction in mitochondrial function in isolated mitochondria exposed to doxorubicin (19, 20, 22–24), the absence of doxorubicin-induced dysfunction in mitochondria isolated from perfused hearts in this study might have been explained by intracardiac factors which actually interfered with the direct effect of doxorubicin on mitochondrial function. To investigate this we incubated mitochondria isolated from normal rat hearts directly with doxorubicin. Even at concentrations 10 times that found to produce acute cardiotoxicity with distinctive reductions in ventricular systolic and diastolic function and intracellular high-energy phosphates, no abnormalities in mitochondrial function were observed after direct incubation with doxorubicin. It should be noted that in the studies reported from other laboratories, the concentrations of doxorubicin required to induce mitochondrial damage were higher than those used in our work. In our studies mitochondria isolated from doxorubicin-perfused hearts had slightly increased rates of States 2, 3 and 4 respiration as compared to control mitochondria. This may represent a mild degree of uncoupling as suggested by previous investigators (22, 23). It is interesting that this was not observed in isolated mitochondria directly incubated with doxorubicin and probably represents an interaction of other intracardiac factors with doxorubicin (possible ischemia as suggested below). In those mitochondria which did demonstrate a slight doxorubicin-induced increase in respiratory rates, there was no decrease in the ADP:O ratio suggesting that, if there were actual uncoupling, it must have been to a minimal degree.

Recently, it has been demonstrated that doxorubicin binds to cardiolipin in the inner mitochondrial membrane and may alter mitochondrial function by excluding such enzymes as cytochrome *c* oxidase and creatine kinase from their membrane binding sites (19–21). These studies have demonstrated reduction in cytochrome *c* oxidase activity in mitochondria directly incubated with doxorubicin (19) and prevention of creatine kinase rebinding to mitochondria and cardiolipin-containing liposomes in the presence of doxorubicin (20, 21). However, overall mitochondrial function was not tested, nor were these observations repeated in intact hearts. In our studies only a minimal reduction in mitochondrial creatine kinase was found in the setting of acute doxorubicin cardiotoxicity. This may be explained by the fact that while doxorubicin prevents rebinding of dissociated creatine kinase, it may not actually cause release of creatine kinase acutely at the doses which we employed. Higher doses, about 1 mM, were required for significant enzyme release (20). Since doxorubicin does bind to cardiolipin in the inner mitochondrial membrane, it may accumulate at that site and contribute to the chronic effects of doxorubicin on cardiac structure and function. The minimal reduction of mitochondrial creatine kinase does not appear to be sufficient to account for the reproducible functional changes.

Since the current study does not support the concept that acute doxorubicin-induced cardiac toxicity results from a direct interference with mitochondrial function, other mechanisms appear to be responsible for the simultaneous reduction in intracellular high-energy phosphate content and ventricular function. The most obvious factor is the marked increase in coronary resistance (9, 10, 12) which might be responsible for a maldistribution of coronary flow with resultant areas of cellular ischemia. By holding coronary flow constant, these studies minimized the LV functional abnormalities caused by doxorubicin. In the intact organism increased coronary resistance and decreased LV function would naturally have led to reduced coronary flow, and by causing ischemia would have magnified the observed alterations in LV function observed in these experiments. While this resultant ischemia could account for the abnormalities in ventricular function and ATP and phosphocreatine content, they might not be severe enough to produce acute mitochondrial damage (36). In fact, moderate degrees of ischemia have been shown to increase State 3

respiration, with glutamate/malate but not succinate as respiratory substrates (37). These changes are identical to those occurring during doxorubicin perfusion, as presented in Tables I and II. However, after repeated drug exposures, ischemia-induced mitochondrial damage might summate and be further compounded by possible accumulation of doxorubicin in mitochondrial membrane cardiolipin. In this manner one might account for the cellular damage seen in the chronic condition of drug administration.

While the present study suggests that ischemic damage may occur during each acute drug exposure, the link to chronic doxorubicin cardiomyopathy is less well established. It is possible that ischemic damage occurring during each exposure accumulates, but it is also possible that an entirely different process is responsible for the chronic cardiomyopathy. Unfortunately, the current model would not be likely to differentiate between these possibilities. However, future studies may establish a link between acute and chronic damage by intervening to reduce acute damage and assessing whether this reduces the development of chronic cardiomyopathy.

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1. Minow RA, Benjamin RS, Gottlieb JA. Adriamycin (NSC-123127) cardiomyopathy—An overview with determination of risk factors. Cancer Chemother Rep **6**:195–201, 1975.
2. Dresdale A, Bonow RO, Wesley R, *et al.* Prospective evaluation of doxorubicin-induced cardiomyopathy resulting from postsurgical adjuvant treatment of patients with soft tissue sarcomas. Cancer **52**:51–60, 1983.
3. Praga C, Beretta G, Vigo PL, *et al.* Adriamycin cardiotoxicity: A survey of 1273 patients. Cancer Treat Rep **63**:827–834, 1979.
4. Bristow MR, Mason JW, Billingham ME, Daniels JR. Doxorubicin cardiomyopathy: Evaluation by phonocardiography, endomyocardial biopsy, and cardiac catheterization. Ann Intern Med **88**:168–175, 1978.
5. Borow KM, Henderson IC, Neuman A, *et al.* Assessment of left ventricular contractility in patients receiving doxorubicin. Ann Intern Med **99**:750–756, 1983.
6. Jaenke RS. An anthracycline antibiotic-induced cardiomyopathy in rabbits. Lab Invest **30**:292–304, 1974.
7. Jaenke RS. Delayed and progressive myocardial lesions after adriamycin administration in the rabbit. Cancer Res **36**:2958–2966, 1976.
8. Singer JW, Narahara KA, Ritchie JL, Hamilton GW, Kennedy JW. Time- and dose-dependent changes in ejection fraction determined by radionuclide angiography after anthracycline therapy. Cancer Treat Rep **62**:945–948, 1978.
9. Vick JA, Herman EH. An isolated dog or monkey heart preparation for studying cardioactive compounds. Pharmacology **6**:290–299, 1971.
10. Herman EH, Mhatre RM, Lee IP, Waravdekar VS. Prevention of the cardiotoxic effects of adriamycin and daunomycin in the isolated dog heart. Proc Soc Exp Biol Med **140**:234–239, 1972.
11. Taylor AL, Bulkley BH. Acute adriamycin cardiotoxicity. Morphologic alterations in isolated perfused rabbit heart. Lab Invest **47**:459–464, 1972.
12. Pelikan PCD, Weisfeldt ML, Jacobus WE, Miceli MV, Bulkley BH, Gerstenblith G. Acute doxorubicin cardiotoxicity: Functional, metabolic, and morphologic alterations in the isolated, perfused rat heart. J Cardiovasc Pharmacol **8**:1058–1066, 1986.
13. Wikman-Coffelt J, Rapcsak M, Sievers R, Rouleau JL, Parmley WW. Verapamil, propranolol, and hydralazine protect against the acute cardiac depression induced by adriamycin. Cardiovasc Res **17**:43–49, 1983.
14. Torti FM, Bristow MR, Howes AE, *et al.* Reduced cardiotoxicity of doxorubicin delivered on a weekly schedule. Assessment by endomyocardial biopsy. Ann Intern Med **99**:745–749, 1983.
15. Ng TC, Daugherty JP, Evanochko WT, Digerness SB, Durant JR, Glickson JD. Detection of antineoplastic agent induced cardiotoxicity by 31-P NMR of perfused rat hearts. Biochem Biophys Res Commun **110**:339–347, 1983.
16. Jackson JA, Rehr RB, Peshock R, Willerson JT, Buja LM, Nunnally RL. Phosphorus nuclear magnetic resonance study of acute adriamycin cardiotoxicity. Circulation **68**:III–67 1983. [Abstract]
17. Ferrans VJ. Overview of cardiac pathology in relation to anthracycline cardiotoxicity. Cancer Treat Rep **62**:955–961, 1978.
18. Olson HM, Young DM, Prieur DJ, LeRoy AF, Reagan RL. Electrolyte and morphologic alterations of myocardium in adriamycin-treated rabbits. Amer J Pathol **77**:439–454, 1974.
19. Goormaghtigh E, Brassiur R, Ruysschaert J-M. Adriamycin inactivates cytochrome c oxidase by exclusion of the enzyme from its cardiolipin essential environment. Biochem Biophys Res Commun **104**:314–320, 1982.

20. Newman RA, Hacker MP, Fagan MA. Adriamycin-mediated inhibition of creatine phosphokinase binding to heart mitochondrial membrane. Biochem Pharmacol **31**:109–111, 1982.
21. Muller M, Moser R, Cheneval D, Carafoli E. Cardiolipin is the membrane receptor for mitochondrial creatine phosphokinase. J Biol Chem **260**:3839–3843, 1985.
22. Gosalves M, Blanco M, Hunter J, Miko M, Chance B. Effects of anticancer agents on the respiration of isolated mitochondria and tumor cells. Eur J Cancer **10**:567–574, 1974.
23. Muhammed H, Ramasarma T, Ramakrishna Kurup CK. Inhibition of mitochondrial oxidative phosphorylation by adriamycin. Biochim Biophys Acta **722**:43–50, 1982.
24. Mailer K, Petering KH. Inhibition of oxidative phosphorylation in tumor cells and mitochondria by daunomycin and adriamycin. Biochem Pharmacol **25**:2085–2089, 1976.
25. Jacobus WE, Evans JJ. Nucleoside diphosphokinase of rat heart mitochondria: Dual localization in matrix and intermembrane space. J Biol Chem **252**:4232–4241, 1977.
26. Moreadith RW, Jacobus WE. Creatine kinase of heart mitochondria: Functional coupling of ADP transfer to the adenine nucleotide translocase. J Biol Chem **257**:899–905, 1982.
27. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem **177**:751–766, 1949.
28. Jacobus WE, Lehninger AL. Creatine kinase of rat heart mitochondria: Coupling of creatine phosphorylation to electron transport. J Biol Chem **248**:4803–4810, 1973.
29. Williamson JU, Schaffer SW, Ford C, Safer B. Contribution of tissue acidosis to ischemic injury in the perfused rat heart. Circulation **53**:I-3–14, 1976.
30. Jacobus WE. Respiratory control and the integration of heart high-energy phosphate metabolism by mitochondrial creatine kinase. Annu Rev Physiol **47**:707–725, 1985.
31. Kobayashi K, Neely JR. Control of the maximum rates of glycolysis in rat cardiac muscle. Circ Res **44**:166–175, 1979.
32. Benjamin RS, Riggs CE, Jr, Bachur NR. Plasma pharmacokinetics of adriamycin and its metabolites in humans with normal hepatic and renal function. Cancer Res **37**:1416–1420, 1977.
33. Benjamin RS, Wiernik PH, Bachur NR. Adriamycin chemotherapy—Efficacy, safety and pharmacologic basis of an intermittent single high-dosage schedule. Cancer **33**:19–27, 1974.
34. Bachur NR. Adriamycin (NSC 123127) pharmacology. Cancer Chemother Rep **6**(3):153–158, 1978.
35. Benjamin RS, Riggs CE, Jr, Bachur NR. Pharmacokinetics and metabolism of adriamycin in man. Clin Pharm Therap **14**:592–600, 1973.
36. Bittl JA, Weisfeldt ML, Jacobus WE. Creatine kinase of heart mitochondria: The progressive loss of enzyme activity during *in vivo* ischemia and its correlation to depressed myocardial function. J Biol Chem **260**:208–214, 1985.
37. Pelikan PCD, Niemann JT, Xia G, Jagels G, Criley JM. Enhancement of mitochondrial oxidative phosphorylation capability by hypoperfusion in isolated perfused rat heart. Circ Res **61**:880–888, 1987.

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