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EFFECTS OF ADRIAMYCIN ON HEART MITOCHONDRIAL FUNCTION IN RESTED AND EXERCISED RATS

LI LI JI* and EDNA W. MITCHELL

Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801, U.S.A.

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Abstract—The effect of Adriamycin® (ADM) administration on heart mitochondria was investigated in rats at rest and after an acute bout of maximal exercise. ADM was given intravenously at a dosage of 8 mg/kg body weight 24 and 1 hr before rats were decapitated. Respiratory functions of the isolated heart mitochondria were measured polarographically with both site 1 (pyruvate-malate and 2-oxoglutarate) and site 2 (succinate) substrates. State 4 (basal) respiration was increased using all substrates in ADM-treated rat hearts compared with non-drug control hearts. The mitochondrial respiratory control index was decreased with ADM, but the reduction was due to an increase in state 4 rather than a decrease of state 3 (ADP-stimulated) respiration. ADM administration abolished an exercise-induced elevation of state 3 respiration using all substrates. There was no significant myocardial oxidative damage of dysfunction as evaluated by lipid peroxidation and antioxidant enzyme activity. Addition of exogenous free radicals to the respiratory medium using hypoxanthine and xanthine oxidase resulted in significant deterioration of mitochondrial function in all parameters measured, but no drug- or exercise-specific patterns of damage were revealed. It is concluded that the current dose of ADM (20% of the established cumulative toxic dose) administered within 24 hr can interfere with normal heart mitochondrial function both at rest and during heavy exercise, but does not elicit overwhelming oxidative damage to the myocardium.

Key words: Antioxidant enzymes; adriamycin; heart; mitochondria; exercise; oxidative damage

Adriamycin® (ADM†, doxorubicin) is an anthracycline antibiotic widely used for treatment of various cancers [1]. Like all anti-tumor drugs, ADM produces a number of side-effects, the most serious being heart damage [2, 3]. It is this cardiotoxicity that limits the doses of ADM that can safely be prescribed clinically. The mechanism of ADM toxicity is not entirely clear. Several hypotheses have been put forward in the past decade, such as calcium overload [4], the accumulation of its metabolite, doxorubicinol [5], and the production of free radicals [6–9]. Among the various well-documented effects of ADM on cardiac function, mitochondria have drawn particular attention because of their involvement in producing free radicals via redox cycling in the electron transport chain, sequestering calcium, and generating ATP to support myocardial contraction. ADM has been shown to decrease respiration of isolated mitochondria in normal and tumor cells by inhibiting NADH-oxidase and succinoxidase [1], to inhibit Ca^{2+}, Na^+/K^+- and H^+-transporting ATP phosphohydrolase [2], and to increase inner membrane permeability to Ca^{2+} by altering mitochondrial thiol status [10]. Furthermore, it has been demonstrated that a high dose of ADM (20 mg/kg) strongly depresses the function of complex I–III and complex IV of the mitochondrial respiratory chain [10].

Adriamycin is metabolized to its reduced form, adriamycinol, primarily in the liver. It is the conversion of adriamycinol to its quinone form that generates superoxide radicals. It has been shown in beef heart that ADM can also accept an electron from the mitochondrial respiratory chain and be converted to ADM free radicals, and subsequently superoxide radicals [7]. The superoxides are converted to hydrogen peroxide and hydroxyl radicals, which can act on the lipid membrane of the mitochondria and cause peroxidative damage. It is possible that the heart is particularly sensitive to this type of damage because it has relatively low levels of superoxide dismutase, catalase and glutathione peroxidase [3]. Therefore, it is important to study antioxidant enzymes as well as lipid peroxidation in the heart after administration of ADM.

Despite the large body of literature dealing with ADM toxicity in heart mitochondria, most of the studies were conducted *in vitro* wherein mitochondria were isolated from hearts at the resting condition. There is a paucity of data regarding the influence of metabolic status of the heart on ADM-elicited toxic effects. It is well known that physical exercise can alter the pharmacokinetics (i.e. absorption, distribution and clearance) of a drug, thereby affecting its bioavailability and potency [11]. ADM is a flow-limited drug; therefore, an increase in blood

* Corresponding author: Li Li Ji, Ph.D., University of Wisconsin, 2000 Observatory Drive 1149, Madison, WI 53706. Tel. (608) 262-7250; FAX (608) 262-1656.

† Abbreviations: ADM, Adriamycin®; AST, aspartate aminotransferase; C, non-drug control; EX, exhaustive exercise; ER, 30-min recovery after exhaustive exercise; GPX, glutathione peroxidase; GR, glutathione reductase; LDH, lactate dehydrogenase; MDA, malondialdehyde; Hx, hypoxanthine; 2-OG, 2-oxoglutarate; P-M, pyruvate-malate; R, rested; RCI, respiratory control index; SOD, superoxide dismutase; Succ, succinate; TBARS, thiobarbituric acid reactive substance; and XO, xanthine oxidase.

perfusion to an organ would increase the unbound drug and hence the extraction of the drug from the blood. During maximal exercise, myocardial blood flow increases proportionally to an augmented cardiac workload up to 300% of that at rest, whereas the decreased hepatic blood flow reduces drug metabolism and clearance [11]. Thus, we hypothesize that exercise may exacerbate ADM toxicity to heart mitochondria at a given dose. Furthermore, most studies of *in vivo* ADM toxicity in the heart have used a very high single dose (15–20 mg/kg body wt), whereas we are more interested in a moderate drug dose representative of a typical clinical prototcol. In the present study, we investigated the acute effect of ADM at ~20% of the established cumulative toxic dose (~37 mg/kg body wt; cf. Ref. 12) on heart mitochondrial respiratory function, lipid peroxidation and antioxidant enzyme systems in rats at rest and after a single bout of maximal exercise. In addition, we employed an exogenous free radical generating system to challenge heart mitochondria, in an attempt to reveal potential damage caused by the drug that was not shown under normal conditions.

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats (age 6 months, weight 280–300 g) were housed individually in a temperature-controlled room (22° and 30% relative humidity) with a dark–light cycle of 12–12 hr. Purina rat chow and tap water were provided *ad lib*. On the day of arrival, the animals were weighed and assigned randomly into ADM and non-drug control (C) groups. Each group of rats was further divided into three categories: rest control (R), exhaustive incremental exercise (EX), and exercise followed by 30-min recovery (ER). Animal body weights were recorded weekly. Because the body weight of the female rats stabilizes at this age, no significant differences in body weight were observed between the various treatment groups (see Results).

Administration of ADM. Adriamycin (doxorubicin) was purchased from Adria Laboratories (Dublin, OH). The standard 50-mg vials of lyophilized powders were reconstituted with sterile physiological saline (0.9% NaCl) to derive a solution at a concentration of 2 mg/mL. A total dose of 8 mg/kg body wt was given to rats in the ADM group with two intravenous injections (4 mg/kg per dose) 24 and 1 hr (see following section for details) prior to the rats being killed. The protocol of drug administration was based on the available data on ADM pharmacokinetics showing that plasma clearance of the drug is triexponential with a half-life of the elimination phase (γ-phase) being ~30 hr [13]. The 24-hr total drug dosage (8 mg/kg body wt) was approximately 20% of the recommended maximal cumulative dose (37 mg/kg, cf. Ref. 12) and comparable with that of a single intravenous bolus injection triweekly in humans [13, 14]. The protocol was essentially identical to the one used by Kanter *et al.* [12] except that they injected the two drug doses with a 2-day interval. Prior to injection, the rat was anesthetized by breathing ether and immobilized by a plastic restrainer with the tail submerged in warm water (45°) to dilate the tail veins. The drug was injected via a tail vein with a 26-G needle. No adverse effect of mild ether breathing was found on heart mitochondrial respiratory properties. Rats in the non-drug (C) group were injected with 2 mL of sterilized saline with an identical procedure.

Exercise protocol. To ensure that the rats could run at the assigned work intensity, all rats participated in a 2-week exercise program to get accustomed to treadmill running. Exercise was performed on a Quinton rodent treadmill equipped with electric shocking grids. During the first week the speed and grade were 10 m/min, 0% grade for 10 min/day, 3 days/week. During the second week, this intensity was increased gradually to 20 m/min, 0% grade for a total of no more than 10 min. This exercise regimen has been shown to have a minimal effect on mitochondrial enzymes [15].

On the day of the mitochondrial measurements, the EX and ER rats in either the ADM or C group were subjected to a single bout of exhaustive, graded running on the treadmill before being killed. Speed and grade were 15 m/min, 10% initially and gradually increased by 5 m/min every 5 min until rats ran at 25 m/min, 10% grade; then speed and grade were increased to 27 m/min, 15%, and continued until rats were exhausted. Exhaustion was determined as the rat being unable to upright itself when placed on its back. Preliminary study indicated that rats could run approximately half an hour at this exercise intensity; therefore, the EX rats in the ADM group received the second dose of drug injection, whereas the EX rats in the C group received saline injection, 30 min before exercise started. The EX groups of rats were killed immediaely after exercise. For the ER rats in ADM and C, drug or saline was injected immediately before exercise started, and rats were allowed 30 min or recovery after exercise prior to being killed. These measures ensured that the second drug dose was consistently administered 1 hr prior to killing the rat. The R rats in ADM and C were killed in the resting state at the same time of the day. One or two rats were killed in random order each day over a 6-week time period.

Tissue preparation. After decapitation, blood samples were collected in heparinized tubes and stored at −80° until assayed for enzyme activity and TBARS. The abdominal cavity of the rat was opened, and the heart was quickly removed. The heart was trimmed of the aorta and other blood vessels, weighed, and placed in an ice-cold mitochondrial isolation medium containing 0.25 M sucrose, 1.0 mM EDTA, 20 mM KCl, and 5.0 mM HEPES (pH 7.4). The volume of the medium was adjusted so that the weight:volume ratio was 1:10. Heart mitochondria were prepared according to a previously described method [15]. Briefly, the heart tissues were minced with scissors and homogenized with a motor-driven Potter–Elvehjem Teflon glass homogenizer at 0–4°. The heart homogenate was first centrifuged at 480 *g* for 5 min at 0°. Supernatant was transferred into a new tube, and the pellets were resuspended in the original homogenizing medium (w/v 1:10) and were centrifuged again at 480 *g* for 5 min. The pellets were discarded and the supernatant

was combined with that from the first centrifugation and then centrifuged at 7700 *g* for 5 min. The pellets that contained mitochondria were suspended in 0.25 M sucrose and 2 mM EDTA for the mitochondrial respiratory measurements and enzyme assays. No detectable activity of mitochondrial matrix enzyme citrate synthase was found in the postmitochondrial supernatant with the described preparation method. A fraction of the 480 *g* supernatant was retained as homogenate with a final weight:volume ratio of 1:20. The homogenates were stored immediately at −80°.

Measurements of mitochondrial respiration. Mitochondrial respiratory function was measured polarographically with a Biological Oxygen Monitor System (model YSI 5300, Yellow Spring Instruments, Columbus, OH) at 30° according to Chance and Williams [16] with some modifications [17]. The respiration medium consisted of 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris–HCl and 30 mM glucose (pH 7.4). After the addition of the air-equilibrated buffer medium and the establishment of a stable base line, 20 μL of mitochondrial suspension (0.2 to 0.4 mg protein) was added to the medium, followed by the addition of various substrates. Substrates used in the present study were 2.0 mM pyruvate/2.0 mM malate (P-M), 4.0 mM 2-oxoglutarate (2-OG) or 4.0 mM succinate (Succ). When succinate was used as respiratory substrate, 2.4 μmol rotenone was included. State 3 respiration was initiated with the addition of 150 nmol ADP. The state 4 and state 3 respirations, as well as the RCI, were defined according to Chance and Williams]16]. The uncoupled state of respiration was measured when 0.6 μmol 2,4-dinitrophenol was added to the established state 4 respiration. All measurements were made in duplicates or triplicates immediately after mitochondria were prepared, and measurements were completed within 3 hr starting at the killing of the rat. There was no significant alteration of the mitochondrial RCI between the first and last measurements within each rat.

In addition to the measurements of basal respiratory function, mitochondria were stressed by exogenous free radicals in order to reveal potential disorder not detectable by the basal method. Superoxide radicals ($O_2^{\cdot-}$) were generated *in vitro* by combining 0.13 U/mL XO (EC 1.1.3.22) and 6 nmol Hx at room temperature under aerobic conditions. These compounds were mixed in an Eppendorf micro test tube, lightly vortexed, and transferred into the mitochondrial respiratory chamber immediately prior to the addition of mitochondria. Duplicates were performed for each rat heart mitochondrial sample.

Enzymes and other biochemical analyses. A fraction of the heart mitochondria or homogenate was frozen and thawed three times to rupture the mitochondrial membrane and to release the total membrane-borne and matrix enzyme activities. Enzyme assays were conducted spectrophotometrically with saturating substrates at optimal temperature. We found no significant change in the measured enzyme activities due to storage up to 2 months at −80°. Activities of SOD (EC 1.15.1.1), catalase (EC 1.11.1.6), GPX (EC 1.11.1.9), and GR (EC 1.6.4.2) were measured according to previously described methods [18]. Succinate dehydrogenase (EC 1.3.99.1) was measured according to Singer [19], LDH (EC 1.1.1.27) activity was determined according to the previously cited methods [15], and AST (EC 2.6.1.1) was measured according to Decker [20]. Myocardial lipid peroxidation was determined by measuring MDA content according to Uchiyama and Mihara [21]. Protein concentration in mitochondria was determined by the Bradford method using bovine serum albumin as the protein standard.

Statistical analysis. A three-way analysis of variance method (MANOVA with repeated measure) was used to analyze the data of mitochondrial respiratory function. The three main treatment effects were ADM, exercise (and recovery), and oxygen free radicals. The remaining data were analyzed with a two-way analysis of variance (ADM and exercise). When a significant treatment effect was indicated, a Scheffe post hoc comparison was performed to test the significance of differences between means. $P < 0.05$ was considered significant.

RESULTS

Heart weight and endurance time. Body weight, heart weight, heart/body weight ratio and endurance time of running to exhaustion in the ADM-treated and non-drug groups of rats at the time they were killed are shown in Table 1. Both heart and body weights appeared to be lower in the ADM group compared with those in the C group; however, the differences did not reach statistically significant levels. Heart/body weight ratio was not affected by drug treatment. A singe bout of exercise had no significant effect on heart or body weights of the rats; therefore, the numbers contained in Table 1 were pooled values of the R, EX and ER rats.

ADM treatment had no significant effect on the endurance time of the rat in the exhaustive, increment bout of treadmill running. The control and ADM-treated rats ran at the assigned work loads for 26.3 ± 1.4 and 24.2 ± 1.2 min, respectively.

Heart mitochondrial respiratory function. Heart mitochondrial function was evaluated by measuring state 4 and state 3 respiration with three different substrates, i.e. P-M, 2-OG and Succ. In the non-drug control group, state 4 respiration was increased significantly in the EX rats with both site 1 (P-M and 2-OG) and site 2 (Succ) substrates, and remained

Table 1. Heart weight, body weight, heart/body weight ratio and endurance time of the rats

	Control (N = 16)	Adriamycin (N = 18)
Heart (g)	0.98 ± 0.02	0.92 ± 0.02
Body (g)	293 ± 4	278 ± 8
Heart/body (g/kg)	3.4 ± 0.07	3.4 ± 0.15
Endurance (min)	26.3 ± 1.4	24.2 ± 1.2

Values are means ± SEM, pooled from rested, exercised and recovery groups of rats.

Table 2. Heart mitochondrial state 4 and state 3 respiration rates and respiratory control index in the rats

	Control			Adriamycin		
	R (N = 13)	EX (N = 6)	ER (N = 7)	R (N= 6)	EX (N = 6)	ER (N = 6)
	[ng $O_2 \cdot min^{-1} \cdot (mg\ protein)^{-1}$]					
State 4						
P-M	17 ± 1	26 ± 3*	24 ± 1*	23 ± 1†	23 ± 2	21 ± 2
2-OG	17 ± 2	28 ± 3*	23 ± 1	24 ± 1†	24 ± 2	24 ± 2
Succ	48 ± 5	71 ± 4*	56 ± 9	75 ± 7†	57 ± 9	71 ± 6†
State 3						
P-M	177 ± 8	214 ± 18*	200 ± 7*	190 ± 8	190 ± 12	185 ± 10
2-OG	120 ± 8	168 ± 16*	136 ± 8	148 ± 6	145 ± 9	136 ± 6
Succ	93 ± 7	126 ± 9*	103 ± 15	136 ± 9†	116 ± 16	135 ± 12†
RCI						
P-M	10.3 ± 0.4	8.8 ± 0.5*	8.5 ± 0.5*	8.4 ± 0.5†	8.7 ± 0.9	9.0 ± 0.8
2-OG	7.9 ± 0.5	6.1 ± 0.2*	5.9 ± 0.3*	6.2 ± 0.2†	6.1 ± 0.4	5.8 ± 0.4
Succ	1.9 ± 0.05	1.8 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	1.9 ± 0.1

Values are means ± SEM. Duplicates or triplicates of measurements were performed for each rat with the numbers of rats per treatment group indicated in parentheses. Substrates used for mitochondrial respiration: pyruvate-malate (P-M), 2 mM-2 mM; 2-oxoglutarate (2-OG), 4 mM; succinate (Succ), 4 mM with 2.4 μM rotenone. Adriamycin (8 mg/kg body wt in two doses) was injected i.v. 24 and 1 hr prior to killing the rats. Abbreviations: R, rested; EX, exhaustive incremental exercise as described in the text; ER, exercise followed by 30 min of recovery; and RCI, respiratory control index.

* $P < 0.05$, EX or ER vs R.

† $P < 0.05$, Adriamycin vs non-drug control.

elevated 30 min after exercise (ER) with P-M (Table 2). State 3 respiration rates were also increased significantly in EX rats with all substrates, compared with R rats. In the ER group, state 3 respiration was higher than the resting values with P-M ($P < 0.05$), but not with 2-OG or Succ as substrates. Heart mitochondrial RCI in the non-drug group showed a significant reduction after an acute bout of exercise ($P < 0.05$), and during recovery with both of the site 1 substrates; however, no significant alteration of RCI was found with site 2 substrate (Succ) either after exercise or recovery (Table 2).

The *in vitro* effect of ADM on heart mitochondrial respiration was evaluated (Fig. 1). When ADM (final concentration 5 μM) was added to mitochondria respiring on P-M, state 4 respiration was increased (not shown) but state 3 respiration was decreased significantly by ~50%. Rotenone (Complex I inhibitor) and dinitrophenol (respiratory uncoupler) had no significant effect on the ADM-inhibited state 3 respiration. When mitochondria were respiring on site 2 substrate succinate (with 2.4 μM rotenone), state 3 respiration was enhanced by ADM (5 μM) These observations suggest that the *in vitro* ADM mitochondrial toxification is primarily on Complex I of the electron transport chain.

As also shown in Table 2, acute ADM administration *in vivo* significantly increased heart mitochondrial state 4 respiration with all three substrates tested in the rested rats. The increments were 35–40% with site 1 substrates ($P < 0.05$) and ~50% with site 2 substrate ($P < 0.05$). ADM increased state 3 respiration with Succ ($P < 0.05$), but state 3 respiration with P-M or 2-OG showed no significant change. As a result, the RCI with site 1 substrates (P-M and 2-OG) were decreased

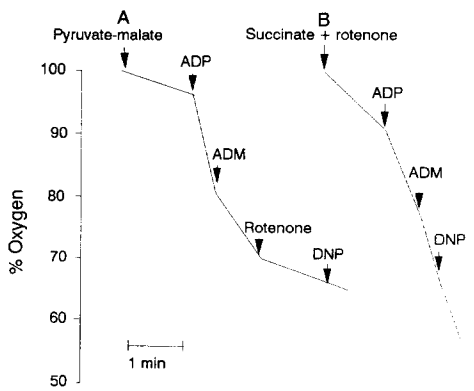

Fig. 1. Effect of Adriamycin (ADM) on heart mitochondria respiring on 2 mM pyruvate + 2 mM malate (A) and 4 mM succinate + rotenone (B), measured in air-equilibrated medium solution consisting of 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris–HCl and 30 mM glucose (pH 7.4) at 30°. Also added into the solution were: mitochondiral protein, 0.2 to 0.4 mg; ADP, 150 nmol; rotenone, 2.4 μmol; and DNP (2,4-dinitrophenol), 0.6 μmol. Final concentration of ADM in the medium, 5 μM.

significantly by ~20% in ADM-administered resting rats, whereas no significant differences in RCI with Succ were present between drug and non-drug groups.

State 4 respiration in ADM-treated exercised rats was not significantly different from that in the ADM-treated resting rats. However, ADM abolished the exercise-caused elevation of state 3 respiration (Table

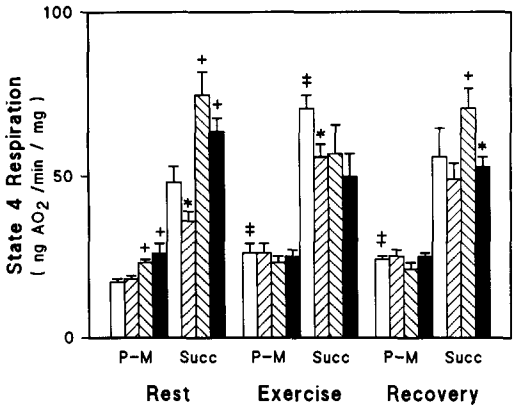

Fig. 2. Effect of oxygen free radicals on heart mitochondrial state 4 respiration in various treatment groups of rats. Open bars: basal rates, non-drug; hatched bars: hypoxanthine (Hx, 6 nmol) and xanthine oxidase (XO, 0.13 U/mL), non-drug; reverse hatched bars: basal rates, Adriamycin (ADM, 8 mg/kg within 24 hr) administered; solid bars: Hx + XO, ADM administered. Key: (*) $P < 0.01$, Hx + XO vs basal with respective substrate; (†) $P < 0.05$, ADM vs non-drug with respective substrate; and (‡) $P < 0.05$, exercise or recovery vs rest group. P-M, 2 mM pyruvate + 2 mM malate; Succ, 4 mM succinate + 2.4 μmol rotenone. Values are means ± SEM with 6–7 rats per group.

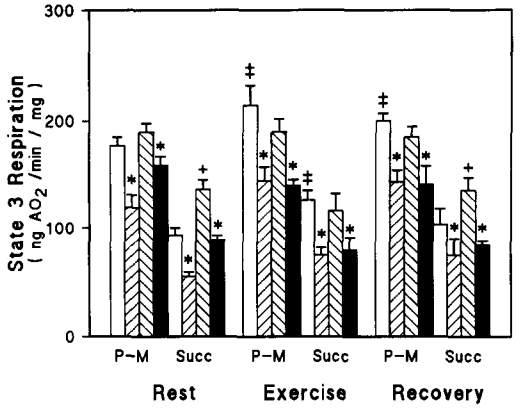

Fig. 3. Effect of oxygen free radicals on heart mitochondrial state 3 respiration in various treatment groups of rats. Open bars: basal rates, non-drug; hatched bars: hypoxanthine (Hx, 6 nmol) and xanthine oxidase (XO, 0.13 U/mL), non-drug; reverse hatched bars; basal rates, Adriamycin (ADM, 8 mg/kg body wt within 24 hr) administered; solid bars: Hx + XO, ADM administered. Key: (*) $P < 0.001$, Hx + XO vs basal with respective substrate; (†) $P < 0.05$, ADM vs non-drug with respective substrate; and (‡) $P < 0.05$, exercise or recovery vs rest group. P-M, 2 mM pyruvate + 2 mM malate; Succ, 4 mM succinate + 2.4 μmol rotenone. Values are means ± SEM with 6–7 rats per group.

2). These findings were true when mitochondria were respiring on either site 1 or site 2 substrates. Thus, ADM appeared to impose a stress-induced increase in basal mitochondrial respiration (state 4) that was essentially equivalent to that induced by maximal exercise, but it hampered the exercise-stimulated activation of respiration in response to ADP.

Susceptibility of heart mitochondria to free radicals. To investigate whether ADM administration altered the susceptibility of heart mitochondria to free radical damage, mitochondria were exposed to external free radicals generated by hypoxanthine and xanthine oxidase. The results are illustrated in Figs. 2–4 with P-M and Succ as substrates. Results similar to those with P-M were obtained with 2-OG as substrate (data not shown).

Free radicals had no significant effect on state 4 mitochondria respiring on P-M with or without ADM administration (Fig. 2). However, State 4 respiration was decreased significantly (20–25%) by Hx + XO when Succ was used as substrate regardless of the drug status ($P < 0.01$). As shown in Fig. 3, free radicals reduced heart mitochondrial state 3 respiration in all treatment groups of rats ($P < 0.001$). The extent of inhibition ranged from 20–30% when P-M were used as substrates to 40–50% when mitochondria were respiring on Succ. ADM administration and the prior metabolic state of the rats did not seem to have a strong influence on the extent of free radical inhibition of mitochondrial state 3 respiration.

Free radicals significantly ($P < 0.001$) decreased heart mitochondrial RCI with site 1 substrates (Fig. 4). The magnitude of reduction appeared to be independent of the drug or the prior metabolic status of the heart. Approximately 35% reduction was

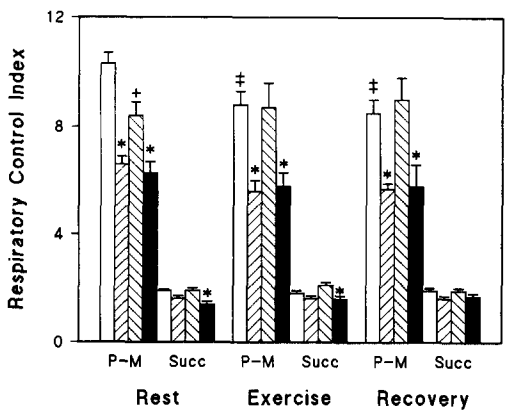

Fig. 4. Effect of oxygen free radicals on heart mitochondrial respiratory control index in various treatment groups of rats. Open bars: basal rates, non-drug; hatched bars: hypoxanthine (Hx, 6 nmol) and xanthine oxidase (XO, 0.13 U/mL), non-drug; reverse hatched bars: basal rates, Adriamycin (ADM, 8 mg/kg within 24 hr) administered; solid bars: Hx + XO, ADM administered. Key: (*) $P < 0.001$, Hx + XO vs basal with respective substrate; (†) $P < 0.05$, ADM vs non-drug with respective substrate; and (‡) $P < 0.05$, exercise or recovery vs rest group. P-M, 2 mM pyruvate + 2 mM malate; Succ, 4 mM succinate + 2.4 μmol rotenone. Values are means ± SEM with 6–7 rats per group.

Table 3. Activity of heart antioxidant enzymes in the rats

	Control			Adriamycin		
	R (N = 6)	EX (N = 6)	ER (N = 6)	R (N = 7)	EX (N = 7)	ER (N = 7)
Mn SOD	8.1 ± 0.4	10.4 ± 1.5*	10.2 ± 1.1*	8.7 ± 1.2	11.7 ± 1.5*	11.8 ± 1.1*
Catalase	67 ± 4.1	59 ± 3.9	58 ± 3.8	65 ± 1.9	61 ± 6.2	61 ± 5.2
GPX	46.2 ± 3.6	51.6 ± 3.1	52.1 ± 1.9	51.2 ± 2.7	52.9 ± 1.5	53.1 ± 3.0
GR	0.6 ± 0.02	0.6 ± 0.03	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.02	0.6 ± 0.02

Values are means ± SEM with the numbers of rats per treatment group indicated in parentheses. Abbreviations: R, rested; EX, exhaustive incremental exercise as described in the text; and ER, exercise followed by 30 min of recovery. Mn SOD, superoxide dismutase measured in mitochondria, U/mg protein. Catalase, measured in heart homogenate, $K \times 10^{-2}$/g wet wt. GPX, glutathione peroxidase, measured in heart homogenate, nmol/min/mg protein. GR, glutathione reductase, measured in heart homogenate, nmol/min/g wet wt.

* $P < 0.05$, EX or ER vs R.

Table 4. Lipid peroxidation and activity of heart enzymes in the rats

	Control			Adriamycin		
	R (N = 6)	EX (N = 6)	ER (N = 6)	R (N = 7)	EX (N = 7)	ER (N = 7)
MDA	1.6 ± 0.1	1.7 ± 0.1	1.4 ± 0.04*	1.6 ± 0.08	1.4 ± 0.08	1.6 ± 0.1
TBARS	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
SDH	603 ± 48	718 ± 56*	636 ± 42	655 ± 40	695 ± 45	566 ± 10
AST	91.4 ± 4	89 ± 5	80 ± 3*	81 + 4	85 ± 2	75 ± 3*
LDH	407 ± 17	398 ± 11	399 ± 8	407 ± 16	405 ± 15	343 ± 11*

Values are means ± SEM with the numbers of rats per treatment group indicated in parentheses. Abbreviations: R, rested; EX, exhaustive incremental exercise as described in the text; and ER, exercise followed by 30 min of recovery. MDA: malonaldehyde, measured in mitochondria, nmol/mg protein. TBARS: thiobarbituric acid reactive substance (A_{530}/mL), measured in mixed arteriovenous blood. SDH: succinate dehydrogenase, measured in mitochondria, nmol/min/mg protein. LDH: lactate dehydrogenase and AST: aspartate aminotransferase, μmol/min/g wet wt, both measured in heart homogenate.

* $P < 0.05$, EX or ER vs R.

independent of the drug or the prior metabolic status of the heart. Approximately 35% reduction was observed in various treatment groups. With site 2 substrate Succ, however, only the ADM group showed a significant reduction of RCI (−25%) with Hx + XO.

Antioxidant enzymes and lipid peroxidation. Heart mitochondrial SOD activity was increased significantly after a single bout of exercise, and remained elevated after recovery in both the non-drug and ADM-administered rats (Table 3). ADM administration appeared to increase SOD activity somewhat, but the change was not significant. Activity of GPX, catalase or GR was not altered by either drug or exercise treatment.

Mitochondrial MDA levels, as an indicator of lipid peroxidation, were not altered by ADM administration or exercise in rats (Table 4). There was no difference in blood TBARS concentration between treatment groups.

Enzymes in substrate metabolism. Mitochondrial SDH was measured as an index of the tricarboxylic acid cycle activity in the heart. SDH activity was increased significantly after an acute bout of exercise, but returned to the resting levels after recovery (Table 4). There was no significant effect of ADM on SDH activity. Activities of AST and LDH, the changes of which have been used previously as indicators of myocardial damage, were not significantly different between the resting and exercise groups. However, AST activity was decreased significantly after recovery ($P < 0.05$) in both the non-drug and ADM groups. ADM-administered rats also tended to have a lower AST activity ($P < 0.07$). The lowest value was found in the ER rats in ADM. LDH activity was significantly lower after recovery ($P < 0.05$) in ADM, but not C rats.

There was no significant difference in myocardial protein content among the various treatment groups (data not shown).

DISCUSSION

There is convincing evidence that ADM cardiotoxicity is caused, at least in part, by generation of oxygen free radicals in the mitochondria [2, 6–9, 22–

24]. It has been shown by careful experiments that the anthracycline can undergo a redox cycling at Complex I of mitochondria and produce $O_2^{\cdot-}$, which, in turn, reacts with its reduction product H_2O_2 to form hydroxyl radicals [8]. It is well established that mitochondrial inner membrane is a primary target of hydroxyl radicals and other reactive oxygen species [25, 26].

Chronic ADM treatment is known to result in a deterioration of heart mitochondrial function [24, 27–29]. There is also sufficient data to support that acute administration of ADM *in vitro* can elicit heart mitochondrial damage including lipid peroxidation, reduction of mitochondrial fluidity, and impairment of oxidative phosphorylation [9, 30]. Consequences of acute ADM administration *in vivo*, however, are less consistent. Some investigators failed to detect ADM-induced dysfunction of heart mitochondria [31], whereas others showed significant damaging effects of ADM on mitochondria morphologically and biochemically [2, 32, 33]. These discrepancies likely reflect difference in drug doses used and the methods to define mitochondrial damage by various investigators. Data from the present investigation demonstrate that ~20% of the cumulative toxic dose of ADM (i.e. 37 mg/kg) administered within 24 hr can induce a significant alteration of heart mitochondrial function. This was revealed by an increase in state 4 respiration with all substrates and a decrease of respiratory control index with site 1 substrates in drug-treated versus non-drug control rats (Table 2). An increase in state 4 respiration was widely regarded as evidence of a leakage of mitochondrial inner membrane [34]. The reduction of RCI provided evidence that the regulation of oxidative phosphorylation in mitochondria was disturbed by drug administration. Although we cannot confirm that oxygen free radicals played a role in the observed changes, these data were consistent with several previous reports showing that oxygen free radicals and their derivatives could cause ultrastructural lesions and biochemical damage to mitochondria [34–36]. Moreover, ADM-treated hearts showed a tendency toward increased SOD activity, which was observed previously in rat heart under oxidative stress [18]. Thus, it is likely that free radical generation was the underlying reason for the observed changes in heart mitochondrial respiratory properties.

Because NADH dehydrogenase (Complex I) in the mitochondrial respiratory chain had been shown to be the major site of free radical generation with ADM [6, 7], it was reasoned that ADM administration would cause a reduction of state 3 respiration when site 1 substrates (pyruvate-malate and 2-oxoglurarate) were used. Our *in vitro* data supported this hypothesis, but *in vivo* data did not. Addition of a micromolar concentration of ADM to mitochondrial respiratory medium resulted in an inhibition of state 3 respiration (Fig. 1). However, there was no significant change in state 3 respiration comparing ADM and non-drug hearts at the resting state. This was most likely related to the limited dose of the drug. The most commonly used clinical dosage schedule is 60–75 mg/m^2 as single intravenous injection administered at a 21-day interval. Alternatively, 30 mg/m^2 on 3 successive days repeated every 4 weeks has been used [14]. In the present study, the cumulative dose of the drug used (8 mg/kg) was lower than most of the other acute animal studies reported in the literature (i.e. 15–20 mg/kg). Although this dose was more relevant to the clinical single dosage used in humans, it might not be sufficiently high to elicit a depression of state 3 respiration, lipid peroxidation or alteration of antioxidant enzyme activity. Alternatively, it is possible that a longer time period might be required for ADM to elicit a toxic effect on heart mitochondria. Doxorubicin and other anthracyclines with C-13 carbonyl functional moieties are metabolized either to the C-13 alcohol metabolite, doxorubicinol, or to deoxydoxorubicin aglycone [13]. It has been demonstrated that in addition to liver, the heart can also convert doxorubicin to doxorubicinol, which is a more potent inhibitor of cardiac function [5]. With a relatively long half-life of elimination (~30 hr), there is a significant accumulation of doxorubicinol in the heart with a typical dosage schedule. This explains the chronic cumulative nature of ADM cardiotoxicity [5]. With the low dosage we used, however, accumulation of doxorubicinol was not likely to occur. Therefore, free radical generation as a mechanism for the observed disturbance of heart mitochondria cannot be discounted without further investigation.

A major finding in the present study was that ADM administration abolished the exercise-induced increase in mitochondrial state 3 respiration with site 1 substrates, whereas it resembled the stress-induced increase in state 4 respiration. In normal hearts, myocardial energy utilization is greatly increased during exercise and mitochondria must be energized to meet this demand. Therefore, an elevation of state 3 respiration was observed in the exercised rats (Table 2). This can be attributed to a number of unconfirmed factors occurring during exercise, including the activation of the enzymes related to substrate dehydrogenation and membrane transport systems for the reducing equivalents (NADH and FADH) [37]. An exercise-caused increase in mitochondrial sequestration of Ca^{2+} may also be involved. The inhibitory effect of ADM on Complex I (NADH dehydrogenase) of the electron transport chain may attenuate the delivery of reducing equivalents and hence the rate of oxidative phosphorylation [10]. Thus, ADM-toxified heart showed a degraded capacity to produce ATP in response to metabolic demand. Our data obtained in the isolated mitochondria seem to be consistent with several previous reports on the effects of ADM on cardiovascular performance during exercise [38, 39].

At the present time, it is unknown whether an increased coronary blood flow during exercise was a contributing factor to the observed drug effects on heart mitochondria. Maximal cardiac work itself is known to cause some adverse effects on ultrastructural and biochemical properties of the heart [40], which may increase the susceptibility of mitochondria to the drug. Exercise can increase tissue extraction of flow-limited drugs, such as ADM, from blood circulation [11]. The decreased hepatic

and renal blood flow during exercise can also prolong the elimination time for the drug, thereby increasing its bioavailability to organs with enhanced blood flow, such as heart and skeletal muscle. Data of the activities of AST and LDH, two enzymes frequently used as indices of myocardial leakage, indicated that ADM-treated rats had a greater loss of myocardial enzymes following exhaustive exercise. Mitochondrial Mn SOD appeared to be activated in exercised ADM rats. These observations suggest that physical exercise may potentiate ADM cardiotoxicity in the heart, possibly due to an altered pharmacokinetics of ADM during exercise.

To test if ADM administration could render the heart mitochondria less defensive against exogenous free radical insult, we exposed the mitochondria from drug- and non-drug-treated hearts to a burst of $O_2^{\cdot-}$ generated by Hx + XO. The effectiveness of this free radical challenge was verified by the significant reduction of state 3 respiration with various substrates used (Fig. 3). However, there were no substantial differences in the magnitude of reduction between the ADM-administered and non-drug control rats, with the exception that when succinate was substrate, RCI was reduced significantly by exogenous free radicals in ADM-treated hearts but not the non-drug controls. This was because Hx + XO inhibited succinate-supported state 3 respiration to a greater extent than state 4 respiration.

In conclusion, *in vivo* administration of ADM at ~20% of the cumulative toxic dose within 24 hr in rats interfered with the normal heart mitochondrial respiratory function, including an increased state 4 respiration and a reduction of respiratory control at rest, and an attentuated state 3 respiration during exercise. However, the administered drug dose did not impair myocardial antioxidant functions, or alter its susceptibility to exogenous free radicals. The apparent interaction between the drug and exercise requires further investigation.

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