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PRELIMINARY COMMUNICATION

DAUNORUBICIN-STIMULATED REACTIVE OXYGEN METABOLISM IN CARDIAC SARCOSOMES*

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Daunorubicin is an anthracycline antibiotic that is now part of standard drug regimens for the therapy of acute myelogenous leukemia in both children and adults [1]. Unfortunately, treatment with this agent is associated with a dose-dependent and potentially lethal congestive cardiomyopathy that limits its use in clinical practice [1]. While the mechanism of daunorubicin cardiac toxicity is incompletely understood, it is now known that several flavin-containing enzymes convert daunorubicin to a semiquinone intermediate that may be responsible for peroxidative injury to cardiac membrane lipids [2 ,3]. This daunorubicin free radical could also reduce molecular oxygen to superoxide anion, beginning a cytotoxic oxygen radical cascade that may be magnified by the heart's limited enzymatic capacity to detoxify highly reactive oxygen species [4]. Because cardiac damage from daunorubicin characteristically includes disruption of the sarcoplasmic reticular membrane [5], we examined the drug's effect on oxygen radical metabolism in a rat heart sarcosomal fraction that has been shown previously to contain sarcoplasmic reticulum [6]. Our results indicate that daunorubicin significantly increases sarcosomal superoxide production, probably as a result of electron transfer by NADPH cytochrome P-450 reductase (E.C. 1.6.2.4). Thus, drug-induced oxygen radical formation may be related to the toxic effect of daunorubicin on cardiac sarcoplasmic reticulum.

Materials and Methods

Male Sprague-Dawley rats weighing 180 to 200 grams were obtained from Mission Laboratory Supply Co., Rosemead, Calif. Daunorubicin hydrochloride of clinical grade was provided by the Investigational Drug Branch, National Cancer Institute, Bethesda, Md. NADPH type III, $NADP^+$, NADH grade III, l-histidine, EDTA, cytochrome c (type VI from horse heart), and bovine erythrocyte SOD (2900 units/mg protein as assayed by the method of McCord and Fridovich [7]) were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium acetate, acetic anhydride, and potassium chloride were purchased from Fisher Scientific Co., Fair Lawn, N.J.

Experimental animals were killed by cervical dislocation; cardiac ventricles were excised, trimmed, blotted dry, and then vigorously washed free of erythrocytes with an iced solution of 100 mM KCl containing 5 mM histidine, pH 7.3. The heart muscle was minced into 25 mg replicates and then homogenized at 4°C for 2 min in 4 volumes of the KCl-histidine buffer with an iced Brinkmann Model

*Abbreviation used in the text:
SOD, superoxide dismutase (Superoxide: superoxide oxidoreductase E.C. 1.15.1.1).

PCU-2-110 Polytron. The rat heart sarcosomal fraction was prepared from the tissue homogenate by differential ultracentrifugation as described by Martonosi [8]. After preparation, the sarcosomal fraction was resuspended in 150 mM potassium phosphate buffer, pH 7.4, containing 100 μM EDTA. Sarcosomal protein concentrations were determined by the method of Lowry et al. [9].

Superoxide formation was determined in paired samples by the SOD-inhibitable reduction of cytochrome c [10]; the cytochrome c utilized had been acetylated previously as described by Azzi et al. [10] in order to eliminate interfering reactions by cytochrome c oxidases or reductases contained in the sarcosomal fraction [10]. The initial linear rate of acetylated cytochrome c reduction was followed spectrophotometrically at 550 nm and 37°C in a Gilford Model 250 recording spectrophotometer equipped with a circulating water bath, using an extinction coefficient for cytochrome c (reduced-oxidized) of 19.6 $mM^{-1}cm^{-1}$ [11]. Paired 1.0 ml control reaction mixtures contained 150 μmoles potassium phosphate buffer, pH 7.4, 100 nanomoles EDTA, 56 nanomoles acetylated cytochrome c, 200 μg sarcosomal protein (prepared daily), and either 0 or 10 μg SOD. Reactions were initiated with 1 μmole NADPH, unless specified otherwise. Where indicated, daunorubicin was included in the paired reaction mixtures. Because the pharmacokinetics of daunorubicin accumulation in heart sarcosomes is, at present, unknown, we chose to use a range of drug concentrations in these experiments that approximates both the peak level of daunorubicin in rat plasma after intravenous administration as well as the apparent Km for daunorubicin free radical formation in hepatic microsomes [12,13].

In related experiments, the rate of NADPH oxidation was determined in triplicate at 340 nm and 37°C in a similar 1.0 ml reaction mixture from which cytochrome c and SOD were eliminated; 100 nanomoles of NADPH was present at the start of the reaction. NADPH consumption was initiated by the addition of the sarcosomal protein, and was calculated using an extinction coefficient of 6.22 x $10^3 m^{-1}cm^{-1}$ [14].

Oxygen consumption was measured at 37°C with a Model 53 oxygen monitoring system from Yellow Springs Instrument Co. The 3.0 ml final reaction mixture contained 450 μmoles potassium phosphate buffer, pH 7.4, 300 nanomoles EDTA, and 1.5 mg sarcosomal protein. Buffers were bubbled with air for at least 30 min at 37°C before use. After equilibration of buffers and sarcosomes for 4 min in the reaction vessel, the electrode was inserted and 3 μmoles NADPH was added; the control rate of oxygen consumption was determined for 10 min thereafter. A similar procedure was used in the presence of 405 nanomoles of daunorubicin, which was added with the sarcosomes. The rate of oxygen consumption was based on a value of 597 nanomoles for the total dissolved oxygen content of the reaction mixture [15]. Data were analyzed with the two-tailed Student's t-test for independent means (NS, not significant, $P > 0.05$ [16]).

Results and Discussion

As seen in Fig. 1A, heart sarcosomes produced a limited degree of SOD-inhibitable acetylated cytochrome c reduction in the absence of daunorubicin. However, in the presence of the drug, Fig. 1B, SOD-inhibitable cytochrome c reduction was substantially increased. Table 1 shows that daunorubicin-enhanced superoxide formation was dose-dependent and was significantly higher than control levels for each daunorubicin concentration tested. Drug-related superoxide production occurred only in the presence of NADPH (and not NADH) as cofactor, was abolished when the sarcosomal protein was denatured by heat, and was reduced over 65% ($P < 0.01$) by the addition of excess $NADP^+$ (Table 1). After denaturation by exposure to heat, the SOD preparation inhibited drug-related cytochrome c reduction much less successfully than the native dismutase (Table 1), strongly suggesting that superoxide production was measured under these experimental conditions. As an independent confirmation

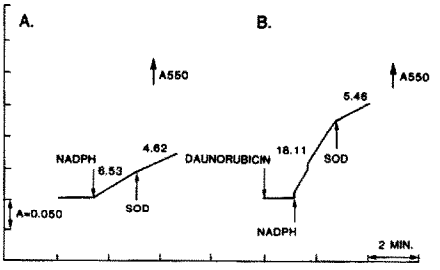

Fig. 1. Effect of daunorubicin on acetylated cytochrome c reduction by cardiac sarcosomes. Addition of NADPH (1 μmole), daunorubicin (135 nanomoles), or SOD (10 μg) indicated by arrows. Numbers above tracings represent the rate of cytochrome c reduction in nanomoles/min/mg.

Table 1. Effect of daunorubicin on superoxide formation in cardiac sarcosomes

Reaction Mixture	Superoxide Formation (nanomoles SOD-inhibitable cytochrome c reduction/min/mg protein ± S.E.M.)	N*
Control	1.33 ± 0.20	8
-Minus NADPH	0.00 ± 0.00	3
-Using NADPH (100 nanomoles)	0.66 ± 0.03	3
-Using NADPH (100 nanomoles) plus $NADP^+$ (1.0 μmole)	0.00 ± 0.26	3
-Minus NADPH plus NADH (1.0 μmole)	0.00 ± 0.00	3
Daunorubicin (10 nanomoles)	2.55 ± 0.13†	3
Daunorubicin (45 nanomoles)	4.81 ± 0.27†	3
Daunorubicin (90 nanomoles)	7.28 ± 0.51†	3
Daunorubicin (135 nanomoles)	8.85 ± 0.31†	6
-Minus NADPH	0.20 ± 0.10	3
-Minus Cytochrome c	0.00 ± 0.00	3
-Minus Sarcosomes	0.26 ± 0.08	3
-Minus NADPH plus NADH (1.0 μmole)	0.00 ± 0.00	3
-Using NADPH (100 nanomoles)	5.71 ± 0.46	3
-Using NADPH (100 nanomoles) plus $NADP^+$ (1.0 μmole)	1.96 ± 0.20	3
-Using Heat-Denatured Sarcosomes‡	0.46 ± 0.23	3
-Using Heat-Denatured SOD‡	7.34 ± 0.33	3

* Number of experiments.

† Significantly higher than control, $P < 0.01$.

‡ Sarcosomes or SOD heated for 60 minutes in a boiling water bath; samples containing denatured SOD paired against identical mixtures with native dismutase.

of these results, we determined that daunorubicin significantly increased oxygen consumption in cardiac sarcosomes (nanomoles O_2/min/mg $\pm$ 1 S.E.M.) from a control rate of 11.38 $\pm$ 0.32 (n=6) to 13.81 $\pm$ 0.40 (n=8) in the presence of the drug, $P < 0.01$. Much of this increase in sarcosomal oxygen consumption may be due to drug-induced superoxide formation.

We have also found that NADPH consumption in sarcosomes was associated with the reactive oxygen metabolism stimulated by daunorubicin. NADPH oxidation (nanomoles NADPH oxidized/min/mg $\pm$ 1 S.E.M.) increased from the 3.27 $\pm$ 0.05 control level to 5.96 $\pm$ 0.37, 6.79 $\pm$ 0.25, and 10.62 $\pm$ 1.48 nanomoles/min/mg after the addition of 45, 90, and 135 nanomoles daunorubicin, respectively, to the sarcosomes; $P < 0.01$ for each drug dose compared to the untreated control.

These experiments suggest that rat heart sarcoplasmic reticulum contains an NADPH oxidizing enzyme that can be inhibited by $NADP^+$ and that is capable of one-electron transfer to both quinone-containing drugs and molecular oxygen; it is probable that this enzyme is NADPH cytochrome P-450 reductase or a very closely related molecule [3]. As shown here, the presence of this enzymatic activity in sarcosomes leads to a nearly seven-fold increase in cardiac superoxide production after daunorubicin treatment; this degree of reactive oxygen formation has been previously demonstrated to have cytotoxic and peroxidative potential in several different mammalian tissues [17]. Reactive oxygen metabolism may be especially damaging to the heart because, compared to other organs, it is poorly protected against an oxygen radical challenge [4].

In summary, we propose that the conversion of daunorubicin to its free radical by heart sarcosomes may explain the sarcoplasmic reticular membrane damage that is a characteristic feature of daunorubicin cardiac toxicity.

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