Anthraacycline Antibiotic-stimulated Superoxide, Hydrogen Peroxide, and Hydroxyl Radical Production by NADH Dehydrogenase

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ABSTRACT

This study investigated the effect of the anthracycline antibiotics on oxygen radical metabolism by cardiac mitochondrial reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase [NADH:(acceptor) oxidoreductase, EC 1.6.99.3]. Superoxide formation by NADH dehydrogenase after anthracycline treatment appeared to follow saturation kinetics with an apparent K_m of 167.3, 73.3, 64.0, or 47.6 μM for doxorubicin, daunorubicin, rubidazone, or aclacinomycin A, respectively. Superoxide formation by NADH dehydrogenase after doxorubicin treatment occurred with a pH optimum of 7.6 and was accompanied by the production of hydrogen peroxide. Furthermore, drug-related hydroxyl radical generation was detected in this enzyme system by the evolution of methane gas from dimethyl sulfoxide. Hydroxyl radical production proceeded only in the presence of superoxide anion, hydrogen peroxide, and trace amounts of iron or a chelate of iron and ethylenediaminetetraacetate and thus was probably the by-product of a transition metal-catalyzed Haber-Weiss reaction. The antitumor agents mitoxantrone and actinomycin D did not significantly enhance reactive oxygen metabolism by NADH dehydrogenase.

These results suggest that the specific activation of the anthracycline antibiotics to free radicals by NADH dehydrogenase leads to the formation of a variety of reactive oxygen species that may contribute to the mitochondrial toxicity of these drugs.

INTRODUCTION

In clinical oncological practice, the usefulness of the anthracycline antibiotics is impaired by their capacity to produce a dose-dependent and potentially life-threatening form of congestive cardiomyopathy (6, 26). Although the pathogenesis of anthracycline cardiac toxicity remains to be explained completely, several recent studies have suggested that drug-induced oxygen-free radicals may play an important role in damaging the heart (14, 31, 32). In particular, oxidation-reduction cycling of the anthracycline quinone catalyzed by certain flavin-containing enzymes might stimulate cardiac-reactive oxygen metabolism so extensively as to overwhelm the limited antioxidant defenses of the myocardial cell (15, 40).

One characteristic feature of the cardiac injury produced by anthracycline treatment is swelling and disruption of mitochondrial membranes (16). Recent investigations by Thayer (44), Pan et al. (34), and our own laboratory (11) have indicated that the proximal portion of the mitochondrial electron-transport chain is capable of catalyzing the flow of electrons from reduced pyridine nucleotides to the anthracycline quinone. We have also shown that free radical formation by this mechanism can exceed mitochondrial mechanisms for oxygen radical detoxification, leading to measurable drug-induced superoxide anion formation by intact cardiac mitochondria (12). Because reactive oxygen species are capable of critically disrupting a wide variety of essential macromolecules (18), oxygen radical metabolism could be involved in anthracycline-related mitochondrial injury.

In these studies, we have pursued our examination of anthracycline-induced mitochondrial oxygen radical metabolism using a crude preparation of cardiac mitochondrial NADH dehydrogenase [NADH:(acceptor) oxidoreductase, EC 1.6.99.3], the presumed enzymatic site for the one-electron reduction of the anthracycline quinone on the electron-transport chain (44). Our results indicate that the anthracycline antibiotics significantly increase superoxide anion and hydrogen peroxide production by NADH dehydrogenase over control levels. Furthermore, drug treatment results in the formation of highly reactive hydroxyl radical species that possess the oxidizing capacity necessary to produce a wide range of potentially cytotoxic effects on the heart (39).

MATERIALS AND METHODS

Materials. Doxorubicin hydrochloride was purchased from Adria Laboratories, Inc., Wilmington, Del. Daunorubicin, rubidazone, aclacinomycin A, and 5-iminodaunorubicin were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Actinomycin D was purchased from Merck Sharp and Dohme, West Point, Pa. Mitoxantrone was graciously supplied by Dr. R. Pocelinko of Lederle Laboratories, Pearl River, N. Y. All drugs were reconstituted in sterile water on the day of preparation, unless indicated otherwise, and were protected from light until used. Cytochrome c (type VI from horse heart), EDTA, rotenone, NADPH (type III), NADH (Grade III), NADP+, NAD+ (Grade V), flavin adenine dinucleotide (Grade III), flavin mononucleotide, sodium succinate, bovine erythrocyte SOD3 [EC 1.15.1.1; 2900 units/mg as assayed by the method of McCord and Fridovich (30)], DMSO (Grade I), Tris, urea, thiourea, L-tryptophan, N-acetylcysteine, and cardiac NADH dehydrogenase (which was free of SOD activity) were purchased from Sigma Chemical Co., St. Louis, Mo. This porcine heart enzyme is a crude preparation of the so-called “low-molecular-weight” NADH dehydrogenase described by Mahler (28); it is a flavin-containing iron-sulfur protein that is essentially devoid of ubiquinone reductase activity but which is capable of electron transfer to a variety of other substrates, including cytochrome c (21). Potassium cyanide, acetic anhydride, sodium acetate, and ferrous sulfate were obtained from Fisher Scientific Co., Fair Lawn, N. J. Dimethylurea and diethylurea were purchased from Aldrich Chemical Co., Milwaukee, Wis.

1 The abbreviations used are: SOD, superoxide dismutase; DMSO, dimethyl sulfoxide.

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Anthracine-enhanced acetylated cytochrome c reduction ranged from 78 to 88% over a concentration span of from 20 to 300 nM doxorubicin. Preliminary experiments also revealed that, as in our previous study (12), no additional inhibition of cytochrome c reduction was found using SOD concentrations above 20 nM/ml. Reactions were initiated by the addition of 0.00 nM of NADH. Chemotherapeutic agents were added to the reaction mixtures, where indicated, before initiation with NADH.

Measurement of NADH Consumption. The effect of doxorubicin on the oxidation of NADH by cardiac NADH dehydrogenase was determined in quadruplicate at 37° by the linear decrease in absorbance at 340 nm using the Gilford spectrophotometer. The 1-mL reaction mixture contained 100 μmol of potassium phosphate buffer, pH 7.6, 100 nM of EDTA, 200 nM of NADH, 3.5 milliunits of the enzyme preparation, and either 0 or 90 nM of doxorubicin. NADH consumption was initiated by the addition of the enzyme preparation and was calculated using an extinction coefficient of 6.22 mmol cm⁻¹ (22).

Oxygen Consumption Assay. The rate of oxygen consumption by NADH dehydrogenase was determined at 37° with a Model 53 oxygen-monitoring system (Yellow Springs Instrument Co., Yellow Springs, Ohio). The 3-mL reaction system contained 300 μmol of potassium phosphate buffer, pH 7.6, that had been bubbled with air for 30 min at 37° before use, 300 nM of EDTA, and 31.2 milliunits of NADH dehydrogenase. After the enzyme and buffer had been equilibrated for 4 min in the reaction vessel, 3 μmol of NADH were added to initiate the reaction; the electrode was inserted, and the linear control rate of oxygen consumption was determined for 10 min. In experiments with antineoplastic agents, drugs were added to the reaction vessel with the enzyme prior to initiation by cofactor. In certain experiments, 5 to 10 μl of specific reagents were added to the reaction chamber through the access slot of the oxygen electrode plunger. The rate of oxygen consumption was calculated from a value of 597 nmol for the total dissolved oxygen content of the reaction mixture (8).

Quantitation of Hydroxyl Radical Formation. Several recent studies have shown that, under appropriate experimental conditions, the hydroxyl radical reacts with DMSO to produce a volatile hydrocarbon gas, CH₄ (38). This reaction has now been used to demonstrate hydroxyl radical formation during such varied biological processes as polymorphonuclear leukocyte phagocytosis (38) and the hepatic microsomal metabolism of short-chain alcohols (7). In this study, the production of the hydroxyl radical by NADH dehydrogenase after treatment with anthracycline antibiotics was measured by the formation of methane from DMSO using a modification of the method of Rine et al. (38). Siliconized 3-ml glass tubes were used in these experiments; the final 2-mL reaction mixture was prepared by the sequential addition to the tubes of 200 μmol of potassium phosphate buffer, pH 7.6, that had been bubbled with air for 30 min at 37° before use, 200 nM of EDTA, 200 μmol of DMSO (or less where specified), 140 milliunits of NADH dehydrogenase (or more where necessary), and the indicated amount of antineoplastic agent. The tubes were rapidly sealed with rubber stoppers, and the experimental reactions were initiated by the injection of 2 μmol of NADH through the stoppers. Subsequently, the samples were vigorously mixed and then incubated, usually for 30 min, at 37° in a shaking water bath; the reaction was terminated by placing the experimental tubes on melted ice. Preliminary experiments indicated that DMSO itself in a concentration of 100 μM had no effect on the enzymatic activity of NADH dehydrogenase. A 200-μl sample of the headspace gas from each tube (total volume, 1 ml) was withdrawn into a Hamilton gas-tight syringe; in every case, sampling proceeded only after vigorous mixing of the headspace gas by the introduction and withdrawal of the syringe plunger at least 10 times. The methane concentration of the 200-μl gas sample was determined using a Varian Model 3700 gas chromatograph (Varian Associates, Palo Alto, Calif.) equipped with a flame ionization detector and a 5'-inch x 6'-ft stainless steel column packed with 80/100 mesh Carbosieve B (Supelco, Inc., Bellefonte, Pa.). Nitrogen was used as the carrier gas at a flow rate of 45 ml/min, and the injector, column, and detector temperatures were 200, 150, and 200°, respectively. Using a 0.100-ppm methane standard (Scott Specialty Gases, Plumsteadville, Pa.), a calibration curve was constructed that was linear over the range of 0.05 to 10 nM of CH₄. The retention time for authentic CH₄ under these conditions was 0.5 min. The methane content of the experimental samples was determined from the calibration curve by comparison of peak heights after the background methane content of the laboratory air had been subtracted.
from each experimental reading; the data were expressed as nmol of CH₄ per 200 µl of headspace gas.

Determination of Kinetic Constants. The data were fitted, and kinetic constants were determined from the direct equation relating reaction velocity to substrate concentration using ELSFIT, a program for extended least-squares data fitting (41).

Statistical Methods. Data were analyzed with the 2-tailed t test for independent means [not significant, p > 0.05 (2)].

RESULTS

Effect of Anthracycline Antibiotics on Superoxide Production by NADH Dehydrogenase. To examine the role of oxygen radical metabolism in anthracycline-induced mitochondrial injury, we investigated the effect of the anthracycline drugs on superoxide anion formation by mitochondrial NADH dehydrogenase. As shown in Table 1, the addition of NADH to a preparation of cardiac NADH dehydrogenase produced only a minimal degree of SOD-inhibitable acetylated cytochrome c reduction. The control rate of superoxide production by NADH dehydrogenase increased at higher enzyme concentrations (Chart 1) but never exceeded 1.21 ± 0.03 (S.E.) nmol/min. The low base-line rate of superoxide production by this mitochondrial enzyme may be explained by its lack of ubiquinone reductase and, potentially, other iron: sulfur proteins, since recent studies indicate that, for submitochondrial particles, two-thirds of the superoxide produced by the electron transport chain comes from the ubiquinone:cytochrome b site (45). However, in the presence of doxorubicin, SOD-inhibitable acetylated cytochrome c reduction initiated by NADH was significantly increased (Table 1). Superoxide production by NADH dehydrogenase after treatment with doxorubicin varied with the hydrogen ion concentration of the incubation system, although drug-related superoxide formation by doxorubicin occurred with little variation between pH 7.0 and 7.8; the optimal pH for oxygen radical production by NADH dehydrogenase was pH 7.6 (Chart 2).

Doxorubicin increased superoxide formation by NADH dehydrogenase in a dose-dependent fashion that appeared to follow saturation kinetics (Chart 3); the apparent $K_m$ and $V_{max}$ for doxorubicin were found to be 167.3 µM and 8.0 nmol/min, respectively. In these studies, drug-enhanced superoxide formation varied with the NADH dehydrogenase activity used (Chart 1) and was abolished when the enzyme preparation was denatured by heat (Table 1). Each component of this experimental system, including cytochrome c, NADH, and the NADH dehydrogenase preparation, was necessary to demonstrate drug-induced oxygen radical metabolism (Table 1), and NADH was the only

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Requirements for doxorubicin-enhanced superoxide formation by NADH dehydrogenase</th>
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<tbody>
<tr>
<td>Experimental system</td>
<td>Superoxide production (nmol reduced/min)</td>
</tr>
<tr>
<td>Control</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Plus rotenone (4 nmol)</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Minus NADH plus NADPH (200 nmol)</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Doxorubicin (90 nmol)</td>
<td>2.54 ± 0.27</td>
</tr>
<tr>
<td>Plus rotenone (4 nmol)</td>
<td>2.14 ± 0.05</td>
</tr>
<tr>
<td>Minus cytochrome c</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Minus NADH</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Minus enzyme</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>
| Using heat-denatured enzyme
d | 0.00 ± 0.00 |
| Using heat-denatured SOD
d | 2.97 ± 0.13 |
| Plus catalase (1500 units) | 2.54 ± 0.10 |
| Plus DMSO (13 µmol) | 2.36 ± 0.03 |

a Mean ± S.E. of 3 to 7 experiments of the rate of superoxide production by NADH dehydrogenase.

b Significantly different from control, p < 0.001.

c No significant difference between results from samples containing rotenone, heat-denatured SOD, catalase, or DMSO and those obtained using doxorubicin alone.

d Enzyme or SOD heated for 60 min in a boiling water bath; samples containing heat-denatured SOD were paired against identical mixtures with native SOD.
cofactor tested capable of supporting superoxide production in this reaction (Table 2). Furthermore, at a constant doxorubicin concentration (250 µM), superoxide production by NADH dehydrogenase appeared to increase as a hyperbolic function of NADH concentration; the apparent K_m for NADH in these experiments was 26.5 µM with a V_max of 7.5 nmol/min (Chart 4).

We also found that SOD-inhibitable cytochrome c reduction was not diminished by DMSO, a potent hydroxyl radical scavenger, or by the addition of sufficient catalase to eliminate hydrogen peroxide from the reaction mixture (Table 1). Finally, the lack of inhibition of doxorubicin-related cytochrome c reduction by denatured SOD indicated that superoxide production had been measured in these experiments (Table 1).

Since other anthracyclines in addition to doxorubicin are known to produce mitochondrial damage in humans (6) and in experimental animals (9), these drugs as well as certain nonanthracycline chemotherapeutic agents were examined for their ability to stimulate superoxide production by NADH dehydrogenase. Daunorubicin, rubidazone, and aclacinomycin A all significantly increased oxygen radical metabolism by the mitochondrial enzyme; kinetic constants for these drugs have been shown in Table 3. However, over the concentration range from 10 to 200 µM, the daunorubicin analogue 5-iminodaunorubicin, which has an imino substitution for the quinone ring oxygen, as well as actinomycin D and the nonanthracycline quinone mitoxantrone did not significantly increase superoxide production over control levels (data not shown).

In related experiments, we found that the stimulation of superoxide production by NADH dehydrogenase after doxorubicin treatment was accompanied by the oxidation of NADH. The addition of doxorubicin (90 µM) to the reaction mixture increased NADH consumption from the control level of 0.03 ± 0.02 (S.E.) nmol/min (n = 4) to 1.80 ± 0.10 nmol/min (n = 4, p < 0.001). Taken together, these experiments strongly suggest that the anthracycline antibiotics significantly enhance the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase.

Effect of Anthracycline Antibiotics on Oxygen Consumption by NADH Dehydrogenase. To investigate the production of hydrogen peroxide by NADH dehydrogenase and the anthracyclines and to confirm our findings using the spectrophotometric assay for superoxide anion, we examined the effect of the anthracycline drugs on the rate of oxygen consumption by NADH dehydrogenase. The experiments were performed as described in Table 1. Points, mean of 3 determinations at each NADH concentration; the doxorubicin concentration used was 250 µM; and the NADH dehydrogenase concentration was 10 milliunits/ml.

Table 3
Kinetic constants for superoxide production by NADH dehydrogenase after treatment with anthracycline antibiotics

<table>
<thead>
<tr>
<th>Drug</th>
<th>K_m (µM)</th>
<th>V_max (nmol/min)</th>
</tr>
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<tbody>
<tr>
<td>Doxorubicin</td>
<td>167.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>73.3</td>
<td>8.1</td>
</tr>
<tr>
<td>Rubidazone</td>
<td>64.0</td>
<td>5.6</td>
</tr>
<tr>
<td>Aclacinomycin A</td>
<td>47.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Kinetic constants were determined using 8 different drug concentrations over a 10-fold concentration range.

Chart 4. Effect of NADH concentration on doxorubicin-stimulated superoxide formation by NADH dehydrogenase. The experiments were performed as described in Table 1. Points, mean of 3 determinations at each NADH concentration; the doxorubicin concentration used was 250 µM; and the NADH dehydrogenase concentration was 10 milliunits/ml.

Table 2
Cofactor requirements for doxorubicin-stimulated superoxide production by cardiac NADH dehydrogenase

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Superoxide formation (nmol cytochrome c reduced/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>8.50 ± 1.85*</td>
</tr>
<tr>
<td>NAD</td>
<td>ND</td>
</tr>
<tr>
<td>NADPH</td>
<td>ND</td>
</tr>
<tr>
<td>NADP</td>
<td>ND</td>
</tr>
<tr>
<td>Flavin adenine dinucleotide</td>
<td>ND</td>
</tr>
<tr>
<td>Flavin mononucleotide</td>
<td>ND</td>
</tr>
<tr>
<td>Succinate</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 3 experiments of the rate of doxorubicin-enhanced superoxide formation by NADH dehydrogenase. ND, none detected (no SOD-inhibitable acetylated cytochrome c reduction found with these cofactors).
cytochrome c reduced oxygen consumption nearly to control levels (Table 4). Under identical experimental conditions, 135 μM doxorubicin and 10.4 milliunits of NADH dehydrogenase per ml, drug-enhanced superoxide production (5.36 ± 0.34, n = 3) was not significantly different from our determination of oxygen consumption after doxorubicin treatment. Thus, a substantial part of the drug-induced increase in oxygen consumption was probably due to the superoxide production demonstrated previously. In this system, the reaction between cytochrome c and superoxide releases oxygen quantitatively, whereas the by-products of the dismutation of superoxide anion are oxygen and hydrogen peroxide. Hydrogen peroxide itself is not measured by the oxygen electrode. This may explain, at least in part, the difference in our results with SOD and cytochrome c. As shown in Table 4, catalase also significantly reduced the rate of oxygen consumption, indicating that hydrogen peroxide was formed under our test conditions. On the other hand, DMSO, a scavenger of the hydroxyl radical, did not alter doxorubicin-stimulated oxygen consumption by NADH dehydrogenase. This was expected because oxygen is not released during the reaction of DMSO with the hydroxyl radical (38). We also found that 5-iminodaunorubicin and mitoxantrone produced no significant increase in oxygen consumption and that treatment with actinomycin D led to a small but significant increase in oxygen consumption by cardiac NADH dehydrogenase (Table 4).

To investigate the stoichiometry of hydrogen peroxide production by NADH dehydrogenase, we performed experiments similar to the one shown in Chart 5A. The release of oxygen by the addition of excess catalase to the reaction vessel indicated, as demonstrated previously, that hydrogen peroxide had been produced in our experiments. Since our enzyme preparation was devoid of SOD and catalase activity as well as the complete components of the hexose monophosphate shunt, for the short time intervals used in these experiments, the H2O2 produced was relatively stable. Because the reaction of catalase with 2 mol of H2O2 yields 1 mol of O2 and 2 mol of H2O, we utilized the method of Biaglow et al. (5) to determine the relationship between H2O2 production and O2 consumption. We found that, in multiple experiments (identical to that shown in Chart 5A), the mean total amount of O2 consumed in a fixed time interval was 54.7 ± 2.7 nmol (n = 4); in the same studies, the mean total H2O2 production (equal to twice the mean catalase-induced O2 release) was 56.52 ± 9.38 nmol (n = 4); thus, there was approximately a 1:1 relationship between H2O2 production and O2 consumption. The experiment shown in Chart 5B suggested that superoxide anion was the direct precursor of the hydrogen peroxide produced by the reaction of doxorubicin with NADH dehydrogenase.

**Generation of Methane from DMSO by Treatment of NADH Dehydrogenase with Anthracycline Antibiotics.** It has been suggested recently that drug-induced hydroxyl radical formation in the heart may be an important mechanism contributing to the cardiac toxicity of the anthracycline antibiotics (13). In many cellular systems, hydrogen peroxide production, such as that demonstrated above, strongly favors the generation of the hydroxyl radical or a hydroxyl radical-like metabolite; thus, we investigated the possibility that the reaction of the anthracycline antibiotics with NADH dehydrogenase could lead to hydroxyl radical formation. We measured the methane released during hydroxyl radical-induced decomposition of DMSO as an indicator of hydroxyl radical generation (38). As shown in Table 5, there was essentially no methane released from DMSO by NADH dehydrogenase in the absence of anthracycline drug. However, in the presence of doxorubicin, there was a dose-dependent increase in methane production that was significantly higher than control levels at all drug concentrations tested (Table 5). Each component of this reaction mixture, including DMSO, NADH, and intact NADH dehydrogenase, was necessary to demonstrate methane production; furthermore, addition of SOD or catalase, but not the heat-inactivated enzymes, completely abolished drug-related methane formation (Table 5). These results indicated that both the superoxide anion and hydrogen peroxide were necessary for the generation of the hydroxyl radical by NADH dehydrogenase. We also found that the anthracycline analogues shown previously to stimulate superoxide formation by NADH dehydrogenase could elicit methane production from DMSO (Table 5).

As shown in Chart 6, the relationship between methane generation and incubation time was not linear for our experimental

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**Table 4**

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>Oxygen consumption (nmol O2/min/ml)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.38 ± 0.08*</td>
</tr>
<tr>
<td>Doxorubicin (405 nmol)</td>
<td>6.55 ± 0.52*</td>
</tr>
<tr>
<td>Plus SOD (60 μg)</td>
<td>4.64 ± 0.72**</td>
</tr>
<tr>
<td>Plus catalase (4500 units)</td>
<td>3.72 ± 0.17**</td>
</tr>
<tr>
<td>Plus SOD (60 μg) and catalase (4500 units)</td>
<td>3.42 ± 0.52**</td>
</tr>
<tr>
<td>Plus acetylated cytochrome c (168 nmol)</td>
<td>0.94 ± 0.06**</td>
</tr>
<tr>
<td>Plus DMSO (60 μmol)</td>
<td>6.34 ± 0.52</td>
</tr>
<tr>
<td>5-iminodaunorubicin (600 nmol)</td>
<td>0.30 ± 0.16</td>
</tr>
<tr>
<td>Mitoxantrone (600 nmol)</td>
<td>0.17 ± 0.15</td>
</tr>
<tr>
<td>Actinomycin D (600 nmol)</td>
<td>0.94 ± 0.06*</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 3 to 9 experiments.
** Significantly different from control, at p < 0.001.
*** Significantly different from samples treated with doxorubicin alone, at p < 0.05.
**** Significantly different from samples treated with doxorubicin alone, at p < 0.001.
***** Significantly different from control, at p < 0.05.
The dismutation of Superoxide anion; hence, in the absence of peroxide produced by NADH dehydrogenase is a by-product of the system. As we have demonstrated previously, the hydrogen...intermediates available in the reaction system; in fact, the rate of methane formation appeared to increase after an initial lag phase. This might be explained if...various oxygen radical intermediates available in the reaction system; in fact, the rate of methane formation appeared to increase after an initial lag phase. This might be explained if...the hydrogen peroxide concentration in the reaction mixture should increase with time. It seems probable that, as has been shown for other biological systems (25), a threshold level of hydrogen peroxide exists that must be present before maximal rates of hydroxyl radical production can be demonstrated.

We also found that methane formation by NADH dehydrogenase after treatment with 135 μM doxorubicin varied with the enzyme concentration studied. At 35, 50, 70, 100, and 350 milliunits of NADH dehydrogenase per ml, methane formation increased from 0.14 ± 0.03 (S.E.) nmol/30 min (n = 5) to 3.71 ± 0.21 (n = 6), 4.44 ± 0.12 (n = 4), 7.53 ± 0.33 (n = 3), and 8.13 ± 0.16 (n = 7) nmol/30 min, respectively. In comparison, at 350 milliunits of NADH dehydrogenase per ml in the absence of doxorubicin, methane production was 0.04 ± 0.02 (n = 4) nmol/30 min, p < 0.001. On the other hand, there was no significant difference in methane formation by NADH dehydrogenase at any DMSO concentration tested; in experiments with 70 milliunits of NADH dehydrogenase and 135 μM doxorubicin, methane formation ranged from 1.68 ± 0.48 nmol/30 min (n = 5) with 5 mM DMSO to 1.74 ± 0.25 (n = 5), 2.08 ± 0.48 (n = 6), and 2.05 ± 0.33 (n = 3) nmol/30 min for 10, 50, or 150 mM DMSO, respectively. Finally, in agreement with the findings of McCord and Day (29), the efficiency of our detecting system for the hydroxyl radical was low; at a doxorubicin concentration of 135 μM and utilizing 50 milliunits of NADH dehydrogenase per ml, the ratio of hydroxyl radical production to superoxide formation was 0.011. This is probably due to a variety of factors which include the multiple reaction products of DMSO with the hydroxyl radical (25) and the fact that the experimental determination of hydroxyl radical production was performed in a gas-tight, closed reaction vessel.

To verify that the evolution of methane from DMSO was a measurement of hydroxyl radical production, we investigated the effect of various hydroxyl radical scavengers on drug-induced methane production by NADH dehydrogenase. We found that thiourea, dimethylurea, diethylurea, n-acetylcysteine, and tryptophan, which are all potent scavengers of the hydroxyl radical (38), competed with DMSO in a concentration-related fashion (Table 6). However, urea, a structurally similar but ineffective hydroxyl radical scavenger (39), had no significant effect on methane production from DMSO (Table 6). Furthermore, we found that each scavenging agent at the highest concentration tested had no effect on the enzymatic activity of NADH dehydrogenase (data not shown). These experiments imply that the reaction of doxorubicin with NADH dehydrogenase leads to the formation of the hydroxyl radical itself or to a hydroxyl radical-like metabolite that reacts similarly with a variety of chemical reagents.

Finally, we investigated the effect of iron and an iron-chelating agent on methane formation in this system. As shown in Table 5, methane production by NADH dehydrogenase was significantly decreased in the absence of EDTA. The addition of 50 μM ferrous sulfate to the identical reaction mixture used for the experiments in Table 5, without EDTA, resulted in no significant change in methane formation, 1.023 ± 0.073 (S.E.) nmol/30 min, n = 4. However, when deferoxamine in a concentration of 50 or 100 μM was added to this experimental system which lacked EDTA, mean methane generation was decreased by 90.4%, 0.10 ± 0.01 nmol/30 min (n = 4, p < 0.001), or 90.2%, 0.11 ±...
the anthracycline quinone. These results lend further support to
microsomal (19) flavin-containing enzymes, NADH dehydrogen
similar to that described previously for both cytoplasmic (33) and
trons from NADH to molecular oxygen by a crude NADH dehy
other anthracycline drugs markedly stimulated the flow of elec
acter complex was the most probable site for the mitochondrial
electron-transport chain (37). This line of investigation was pur
0.01 nmol/30 min (n = 4, p < 0.001), respectively, without
ponent of the mitochondrial dehydrogenase (24). In a process
cardiac NADH dehydrogenase, a flavin-containing enzyme that
DISCUSSION

In this study, we have attempted to characterize the reaction of
the hypothesis that the free radical metabolism of the anthracy
cline drugs occurs early in the mitochondrial respiratory chain.
From our studies and those of other investigators (4, 44), it is
probable that the primary event in the reaction sequence leading
to oxygen radical formation is the reduction of the anthracycline
quinone by NADH dehydrogenase in the presence of reduced cofactor. Substantial evidence for the generation of an anthracy
cline semiquinone intermediate by flavin-containing dehydro-
genases has been published by several laboratories (4, 23), and we
have demonstrated recently typical doxorubicin semiquinone spectra by electron spin resonance using cardiac submitochond-
particles (10). In our aerobic system used for the current
studies, the doxorubicin semiquinone rapidly donated its un-
paired electron to molecular oxygen, forming the superoxide
Anion. It has been suggested by Kalyanaraman et al. (23) that
this reaction would proceed rapidly even at moderate oxygen
tensions and certainly at those present in the heart. In our
experiments, a comparison of the rates of superoxide formation
and oxygen consumption by NADH dehydrogenase under similar
experimental conditions indicated that, at least initially, super-
oxide anion production accounted for most of the oxygen con-
sumed.

In addition to superoxide anion, we found that hydrogen peroxide was formed by NADH dehydrogenase after anthracy-
cline treatment. Because our enzyme preparation lacked SOD
activity, hydrogen peroxide production must have resulted from
the spontaneous dismutation of superoxide anion. Although spontaneous dismutation \( [k = 2 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}] \) is known
occurred at a much slower rate than the SOD-catalyzed reaction
\( [k = 2 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}] \), our experiments suggested that the
dismutation process was complete, yielding an approximate
1:1 stoichiometry between hydrogen peroxide production and
oxygen consumption.

Oxygen radical metabolism by NADH dehydrogenase after
exposure to anthracycline drugs also appeared to involve the
generation of another potent oxidant, the hydroxyl radical (39).
We have shown in this study that methane evolved during the
reaction of NADH dehydrogenase with anthracycline antitumor
agents in the presence of DMSO, a scavenger of the hydroxyl
radical (39). This reaction has been used previously to measure
hydroxyl radical production specifically in both chemical and
biological systems (38, 39). In our hands, methane generation
from DMSO varied with the concentration of enzyme or doxor-
ubicin examined, was significantly reduced after addition of an
avid iron-chelating agent, defereroxamine, and was completely
abolished by SOD or catalase but not the heat-inactivated en-
zymes. These results suggested that superoxide anion, hydro-
gen peroxide, and trace amounts of a transition metal were all
needed for hydroxyl radical production by NADH dehydrogenase
after anthracycline treatment. Thus, it is very probable that
hydroxyl radical formation in our experiments proceeded by way
of the so-called "metal-catalyzed Haber-Weiss reaction" in which
iron or iron-chelates maintained in the reduced state by super-
oxide anion are capable of reacting with hydrogen peroxide
to form the hydroxyl radical (29). This mechanism is supported by
the finding that methane formation was significantly reduced in
the absence of EDTA, since it is now known that iron chelated
with EDTA is more easily capable of participating in these oxi-
dation-reduction reactions (20).

The source of the transition metal supporting this reaction has
the most probable site for the mitochondrial activation of the anthracy
cline and flavin-containing enzymes. NADH dehydrogen-
ase appeared to catalyze the cyclic oxidation and reduction of the anthracycline quinone. These results lend further support to

### Table 6
**Effect of hydroxyl radical scavengers on the production of methane from DMSO by NADH dehydrogenase after treatment with doxorubicin**

<table>
<thead>
<tr>
<th>Test conditions</th>
<th>Methane production (nmol/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>4.32 ± 0.28*</td>
</tr>
<tr>
<td>Plus urea (5 mw)</td>
<td>3.98 ± 0.29*</td>
</tr>
<tr>
<td>Plus urea (15 mw)</td>
<td>4.29 ± 0.42*</td>
</tr>
<tr>
<td>Plus urea (50 mw)</td>
<td>3.65 ± 0.14*</td>
</tr>
<tr>
<td>Plus thioctic acid (5 mw)</td>
<td>2.40 ± 0.15*</td>
</tr>
<tr>
<td>Plus thioctic acid (15 mw)</td>
<td>0.80 ± 0.16*</td>
</tr>
<tr>
<td>Plus thioctic acid (50 mw)</td>
<td>0.03 ± 0.01*</td>
</tr>
<tr>
<td>Plus dimethylurea (5 mw)</td>
<td>4.12 ± 0.43*</td>
</tr>
<tr>
<td>Plus dimethylurea (15 mw)</td>
<td>2.63 ± 0.55*</td>
</tr>
<tr>
<td>Plus dimethylurea (50 mw)</td>
<td>1.25 ± 0.31*</td>
</tr>
<tr>
<td>Plus diethylurea (5 mw)</td>
<td>4.68 ± 0.23*</td>
</tr>
<tr>
<td>Plus diethylurea (15 mw)</td>
<td>2.98 ± 0.36*</td>
</tr>
<tr>
<td>Plus diethylurea (50 mw)</td>
<td>1.68 ± 0.09*</td>
</tr>
<tr>
<td>Plus tryptophan (1 mw)</td>
<td>4.84 ± 0.26*</td>
</tr>
<tr>
<td>Plus tryptophan (5 mw)</td>
<td>3.64 ± 0.07*</td>
</tr>
<tr>
<td>Plus tryptophan (15 mw)</td>
<td>0.19 ± 0.06*</td>
</tr>
<tr>
<td>Plus N-acetylcysteine (5 mw)</td>
<td>1.32 ± 0.19*</td>
</tr>
<tr>
<td>Plus N-acetylcysteine (50 mw)</td>
<td>0.12 ± 0.01*</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 4 to 12 experiments.

** Mean ± S.E. of 4 to 12 experiments.

---

From our studies and those of other investigators (4, 44), it is
probable that the primary event in the reaction sequence leading
to oxygen radical formation is the reduction of the anthracycline
quinone by NADH dehydrogenase in the presence of reduced cofactor. Substantial evidence for the generation of an an-
tracycline semiquinone intermediate by flavin-containing dehydro-
genases has been published by several laboratories (4, 23), and we
have demonstrated recently typical doxorubicin semiquinone spectra by electron spin resonance using cardiac submitochond-
particles (10). In our aerobic system used for the current
studies, the doxorubicin semiquinone rapidly donated its un-
paired electron to molecular oxygen, forming the superoxide
Anion. It has been suggested by Kalyanaraman et al. (23) that
this reaction would proceed rapidly even at moderate oxygen
tensions and certainly at those present in the heart. In our
experiments, a comparison of the rates of superoxide formation
and oxygen consumption by NADH dehydrogenase under similar
experimental conditions indicated that, at least initially, super-
oxide anion production accounted for most of the oxygen con-
sumed.

In addition to superoxide anion, we found that hydrogen peroxide was formed by NADH dehydrogenase after anthracy-
cline treatment. Because our enzyme preparation lacked SOD
activity, hydrogen peroxide production must have resulted from
the spontaneous dismutation of superoxide anion. Although spontaneous dismutation \( [k = 2 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}] \) is known
occurred at a much slower rate than the SOD-catalyzed reaction
\( [k = 2 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}] \), our experiments suggested that the
dismutation process was complete, yielding an approximate
1:1 stoichiometry between hydrogen peroxide production and
oxygen consumption.

Oxygen radical metabolism by NADH dehydrogenase after
exposure to anthracycline drugs also appeared to involve the
generation of another potent oxidant, the hydroxyl radical (39).
We have shown in this study that methane evolved during the
reaction of NADH dehydrogenase with anthracycline antitumor
agents in the presence of DMSO, a scavenger of the hydroxyl
radical (39). This reaction has been used previously to measure
hydroxyl radical production specifically in both chemical and
biological systems (38, 39). In our hands, methane generation
from DMSO varied with the concentration of enzyme or doxor-
ubicin examined, was significantly reduced after addition of an
avid iron-chelating agent, defereroxamine, and was completely
abolished by SOD or catalase but not the heat-inactivated en-
zymes. These results suggested that superoxide anion, hydro-
gen peroxide, and trace amounts of a transition metal were all
needed for hydroxyl radical production by NADH dehydrogenase
after anthracycline treatment. Thus, it is very probable that
hydroxyl radical formation in our experiments proceeded by way
of the so-called "metal-catalyzed Haber-Weiss reaction" in which
iron or iron-chelates maintained in the reduced state by super-
oxide anion are capable of reacting with hydrogen peroxide
to form the hydroxyl radical (29). This mechanism is supported by
the finding that methane formation was significantly reduced in
the absence of EDTA, since it is now known that iron chelated
with EDTA is more easily capable of participating in these oxida-
tion-reduction reactions (20).

The source of the transition metal supporting this reaction has

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not been determined specifically in these experiments. However, the treatment of our phosphate buffers and the NADH dehydrogenase with Chelex 100 significantly reduces the chance that an unbound transition metal contaminant has been introduced with these reagents in a systematic fashion. Iron contamination of our chemotherapeutic agents, on the other hand, has not been excluded. Furthermore, since cardiac tissue contains an abundance of iron-containing proteins that could be degraded during preparation of the enzyme, our NADH dehydrogenase could itself have donated trace amounts of protein-bound iron suitable for catalytic purposes but not removed by treatment with Chelex 100. This may be important because protein-bound forms of iron have been shown recently to be more effective than iron:EDTA chelate in promoting hydroxyl radical formation (1). We could also hypothesize that cardiac tissue might be especially susceptible to a similar reaction sequence if anthracycline administration led to myocardial hydrogen peroxide accumulation in vivo.

In addition to the mechanism of hydroxyl radical formation, we have attempted to examine the specificity of our hydroxyl radical detection system. Five different compounds that share the property of rapidly reacting with the hydroxyl radical have each been shown to reduce methane production from DMSO in a concentration-related manner. At least one of these chemicals, N-acetylcysteine, has been demonstrated to reduce doxorubicin cardiac toxicity in the mouse (14). Urea, a structurally related compound without significant hydroxyl radical-scaevenging properties (38), produced no effect on methane formation at any concentration tested. These results suggest that the hydroxyl radical or a hydroxyl radical-like metabolite with similar chemical reactivities was formed by the interaction of NADH dehydrogenase with the anthracycline antibiotics.

The importance of the oxidation-reduction cycle initiated by anthracycline treatment of mitochondrial NADH dehydrogenase is related, in part, to the possibility that drug-induced oxygen radical metabolism in vivo might overwhelm the limited enzymatic capacity of heart muscle to detoxify free radicals (15, 40). Thus, drug-induced hydroxyl radical production could lead to the peroxidation of mitochondrial membranes and the oxidation of critical sulfhydryl-containing enzymes with a subsequent loss of control of mitochondrial calcium transport (27, 42). Furthermore, reduction of the anthracycline quinone at a proximal site on the electron-transport chain might divert electrons from energy production, leading to diminished contractile function. Hence, unchecked, drug-induced oxygen radical production could explain changes in cardiac mitochondrial morphology and function that have been consistently demonstrated following anthracycline treatment in humans and in experimental animals (26).

Finally, we examined the interaction of other nonanthracycline antineoplastic agents with NADH dehydrogenase to determine the generality of drug activation by this mitochondrial enzyme. It was apparent from these studies that the substrate specificity for quinone reduction by mitochondrial NADH dehydrogenase was more rigid than that for microsomal NADP+-cytochrome P-450 reductase (4). Actinomycin D and mitoxantrone increased oxygen radical production by NADH dehydrogenase to a minor degree or not at all at drug concentrations which for the anthracyclines produced a significant enhancement in reactive oxygen production. Of the anthracycline drugs tested, only 5-iminodaurubicin, which has a substituted quinone ring, did not significantly increase superoxide production by NADH dehydrogenase. Thus, the specific nature of anthracycline activation by NADH dehydrogenase, if confirmed, might be an important determinant of the mitochondrial toxicity of the anthracycline antibiotics.

In summary, we have found that the anthracycline antibiotics as a class share the capacity to stimulate reactive oxygen metabolism by mitochondrial NADH dehydrogenase; the generation of a free radical cascade with the potent oxidizing potential of the hydroxyl radical by these drugs may be an important factor contributing to their myocardial toxicity.

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Anthracycline Antibiotic-stimulated Superoxide, Hydrogen Peroxide, and Hydroxyl Radical Production by NADH Dehydrogenase

James H. Doroshow


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