Mitochondrial NADH dehydrogenase-catalyzed oxygen radical production by adriamycin, and the relative inactivity of 5-iminodaunorubicin

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1. INTRODUCTION

The anthracyclines adriamycin (ADR, doxorubicin) and daunorubicin are antineoplastic antibiotics with substantial therapeutic activity against a wide variety of human malignancies, including cancers of the breast, ovary and the lymphatic system [1]. Unfortunately, the total dose of these agents that can be administered is severely limited by a unique type of cardiac toxicity which may be the direct result of drug-induced free radical formation [2,3]. Both cardiac sarcosomes [2] and mitochondria [2,3] can reduce ADR and daunorubicin to their respective semiquinones, initiating a free radical cascade which heart tissue, with its limited antioxidant defenses [4], seems unable to resist. Thayer [3] suggested initially that NADH dehydrogenase was the mitochondrial site of ADR reduction; however, a detailed study of this proposal as well as an investigation of the range of drug-related oxygen radical metabolism by mitochondria has not previously been conducted. Furthermore, a new anthracycline derivative, 5-iminodaunorubicin (5-IDR), with promising antitumor activity [5] and distinctly different redox properties from those of ADR [5,6] has been synthesized as a potentially non-cardiotoxic anthracycline analogue for use in cancer chemotherapy. Hence, in this study we have attempted to define both the site of anthracycline reduction in the electron transport chain, and the nature and extent of reactive oxygen production stimulated by ADR and 5-IDR.

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Abbreviations: BH-SMP, beef heart submitochondrial particles (preparations); ADR, adriamycin (doxorubicin); 5-IDR, 5-iminodaunorubicin; $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical

2. MATERIALS AND METHODS

Beef heart submitochondrial particles (BH-SMP) were prepared by established procedures [7]. Oxygen consumption was measured polarographically [2,8,9], and NADH oxidation was assayed spectrophotometrically by absorbance decrease at 340 nm [8,9]. Reduction of exogenous NAD^+ to NADH during BH-SMP reverse electron transport was energized by ATP, using succinate as the electron source, in the presence of an antimycin A block [9]. Anthracycline radicals were detected by EPR spectroscopy (Varian E-3) at 25°C, and the spin trap, 5,5-dimethylpyrroline-*N*-oxide (DMPO) was used to estimate hydroxyl radical ($OH^{\cdot}$) production [10]. The superoxide

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radical was measured as superoxide dismutase-inhibitable, acetylated cytochrome *c* reduction [2]. Hydrogen peroxide was determined by the return of oxygen, to a closed system, following catalase addition [2]. ADR was purchased from Adria Labs (Columbus OH) and 5-IDR was supplied by the Drug Synthesis and Chemistry Branch, National Cancer Inst. (Bethesda MD).

3. RESULTS

Addition of ADR to BH-SMP respiring on NADH (in the absence of rotenone) caused a 15–20% decrease in maximal rates of O_2 consumption and NADH oxidation; 5-IDR, however, was without significant effect (table 1). In the absence of anthracycline, rotenone decreased BH-SMP NADH oxidation and O_2 consumption to only 2–3% of the non rotenone-inhibited rates. Addition of ADR to rotenone-inhibited BH-SMP increased the rates of NADH oxidation and O_2 consumption dramatically, but 5-IDR was, again, without effect. These results were not altered by the addition of KCN; and succinate could not substitute for NADH as the electron source. Piericidin A or amytal produced essentially the same results as did rotenone. The same trends were also seen with freshly prepared, intact, mitochondria respiring on pyruvate plus malate (not shown).

The data of table 1 indicate that ADR undergoes reduction via a component of NADH dehydrogenase (mitochondrial complex I) and subsequently reduces molecular oxygen (accounting for the O_2 consumption in the presence of rotenone, piericidin A, amytal or KCN). If this hypothesis is correct, ADR should also be reduced by NADH dehydrogenase during ATP-energized reverse electron transport from succinate to NAD^+. Indeed, in table 2, it is seen that ADR inhibited NAD^+ reduction during reverse electron transport by some 70%, and that the process consumed oxygen. In contrast, 5-IDR had only a minor effect on NAD^+ reduction and did not induce O_2 consumption. NAD^+ reduction was completely inhibited by rotenone or thenoyltrifluoroacetone, and these agents also blocked the ADR-induced O_2 consumption. Furthermore, KCN did not decrease O_2 consumption produced by ADR, thus ruling out a possible electron-transport chain bypass to cytochrome oxidase.

That ADR undergoes a one-electron reduction by mitochondrial NADH dehydrogenase was demonstrated by the production of an ADR free radical (fig.1). The ADR radical ($g \simeq 2.004$, peak to peak signal width $\simeq 4.5$ G) exhibited no hyperfine structure, and was more stable under anaerobic than aerobic conditions. No 5-IDR radical could be discerned at 0.1, 0.2 or 0.3 mM drug concentration; however, a weak signal was evident at 0.5 mM 5-IDR (< 10% of that seen with 0.2 mM ADR).

In the presence of ADR and NADH, rotenone-inhibited BH-SMP produced $O_2^{\cdot -}$, H_2O_2, and $OH^{\cdot}$ (table 3). The production of all three activated ox-

Table 1

Effects of anthracyclines on NADH respiration by beef heart SMP

Anthracycline added	O_2 consumption[a]		NADH oxidation[b]	
	− Rotenone	+ Rotenone	− Rotenone	+ Rotenone
None	268 ± 13	4 ± 1	64 ± 7	2 ± 1
Adriamycin	226 ± 12	116 ± 8	53 ± 9	30 ± 4
5-Iminodaunorubicin	259 ± 11	9 ± 2	62 ± 9	3 ± 1

[a] nmol $O_2 \cdot min^{-1} \cdot mg$ BH-SMP^{-1} at 37°C
[b] nmol NADH $\cdot min^{-1} \cdot mg$ BH-SMP^{-1} at 23°C

Results are V_{max} values (mean ± SE) obtained by Lineweaver–Burk extrapolation (anthracycline range 0–0.6 mM, NADH range 0–2 mM) in 0.1 M KPO_4 buffer (pH 7.2) ± 0.01 mM rotenone

Table 2

Effects of anthracyclines on reverse electron transport from succinate to NAD^+ by BH-SMP

Anthracycline added	NADH production	Oxygen consumption
None	38 ± 3	2 ± 1
Adriamycin	11 ± 1	50 ± 6
5-Iminodaunorubicin	30 ± 2	3 ± 2

Values are nmol NADH produced or O_2 consumed . min^{-1} . mg BH-SMP^{-1} (mean ± SE). Temperature was 23°C for NADH and 37°C for O_2. Results obtained in 0.1 M KPO_4 (pH 7.2), with 10 mM succinate, 0.04 mM antimycin A, and 5 mM ATP, ± 0.2 mM anthracycline

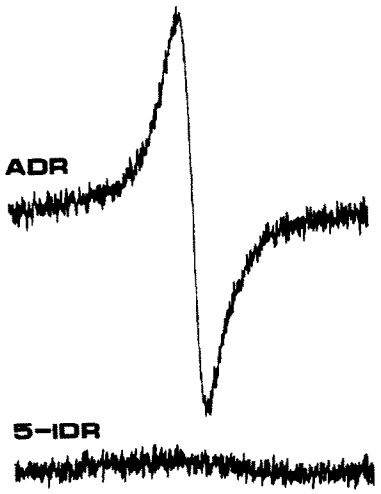

Fig.1. EPR spectra of ADR and 5-IDR incubated with BH-SMP. ADR and 5-IDR were 0.2 mM in 0.1 M KPO_4 (pH 7.2), plus 10 mM NADH and 0.01 mM rotenone. EPR conditions: 100 kHz field; 9.45 GHz microwave frequency; 3 mW microwave power; 4 G modulation; 0.3 s time constant; 8 min scan time; 50 G scan range; at 25°C.

ygen species was inhibited by superoxide dismutase (not shown). Conversely, rotenone-inhibited BH-SMP supplemented with NADH and 5-IDR produced barely detectable levels of $O_2^{\cdot -}$ or $OH^{\cdot}$ ($\simeq$ 7% of that seen with ADR), and no detectable H_2O_2.

Table 3

Superoxide, hydrogen peroxide, and hydroxyl radical production by rotenone-inhibited beef heart SMP

Anthracycline added	Superoxide[a]	Hydrogen peroxide[b]	Hydroxyl radical[c]
None	0 ± 0	0.26 ± 0.26	0 ± 0
Adriamycin	67 ± 3	1.34 ± 0.24	116 ± 9
5-Iminodaunorubicin	5 ± 1	0.00 ± 0.00	8 ± 1

[a] nmol acetylated cytochrome *c* reduced . min^{-1} . mg BH-SMP^{-1} at 37°C

[b] Percent of total O_2 consumed which was returned to system by 1500 units/ml catalase addition

[c] Relative EPR signal height (arbitrary units) of DMPO-$OH^{\cdot}$ adduct at 25°C

Results (mean ± SE) obtained in 0.1 M KPO_4 (pH 7.2) with 0.2–2.0 mM NADH, 0.02–0.10 mM rotenone, and 0.2 mM anthracycline

4. DISCUSSION

Our results demonstrate that a component of mitochondrial NADH dehydrogenase actively reduces ADR, whereas 5-IDR is relatively unreactive. The reaction sequence for ADR appears to be an initial one-electron reduction to produce an ADR$^{\cdot}$ radical. The drug-based free radical then reduces molecular oxygen, producing $O_2^{\cdot -}$ and regenerating the oxidized ADR, establishing an ADR redox cycle. Some of the $O_2^{\cdot -}$ so produced undergoes dismutation to yield H_2O_2. Hydroxyl radical detected under our experimental conditions may be the result of an iron-catalyzed Haber–Weiss reaction, which could also produce singlet oxygen [11]. We propose that a similar free-radical cascade is responsible for the marked toxicity of ADR for cardiac mitochondrial membranes [1].

5-IDR appears to be poorly reduced by mitochondrial NADH dehydrogenase and, as might be expected from previous chemical studies [5,6], has a very limited tendency to auto-oxidize. Thus, unlike ADR, 5-IDR does not participate to any significant extent in NADH dehydrogenase-mediated free radical redox cycles. This is probably due to the imine substitution on the quinone ring. These findings are supported by data sug-

gesting that 5-IDR is poorly reduced by liver microsomes [5], which actively redox cycle ADR and daunorubicin. Since 5-IDR retains antitumor activity against P388 leukemia while inhibiting DNA and RNA synthesis in L1210 cells [6], 5-IDR could prove to be an effective, non-cardiotoxic alternative to other currently available anthracycline anticancer agents.

Previous work with the anthracyclines has largely concentrated on free radical generation by the NADPH cytochrome P450 reductase system of liver endoplasmic reticulum (microsomes) and cardiac sarcoplasmic reticulum (sarcosomes) [12–14]. While such studies have provided extremely valuable models, it is now appropriate to begin a careful assessment of the relative contributions of cardiac mitochondria and sarcoplasmic reticulum to anthracycline toxicity. Specific activities for ADR reduction and $O_2^{\cdot -}$ production were found to be $\geq 100\%$ higher for (intact) cardiac mitochondria than for cardiac sarcosomes, in a direct comparison [2]. Using identical methods, specific activities for oxygen radical production by BH-SMP here are substantially higher than those found for either heart mitochondria or sarcosomes in [2]. This, coupled with the high mitochondrial content, and the NADH/NADPH ratio of cardiac muscle in vivo [15], suggests that drug-related free radical formation by mitochondrial NADH dehydrogenase may play a major role in the etiology of anthracycline cardiotoxicity.

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