GENERATION OF FREE RADICALS AND LIPID PEROXIDATION BY REDOX CYCLING OF ADRIAMYCIN AND DAUNOMYCIN

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Summary: NADPH and purified P450 reductase cause oxygen consumption from the antitumor drugs daunomycin or adriamycin in excess of the amount of the drug present. A reduction-autoxidation cycle of quinone groups is postulated. During this cycle, the cooxidation of sulfite may be initiated. This latter oxidation is inhibited by superoxide dismutase, suggesting that superoxide radicals are formed. Hydrogen peroxide is also generated, presumably by non-enzymatic dismutation of superoxide. Rat liver microsomes also catalyze this redox cycling which is accompanied by the peroxidation of lipids. The experiments suggest that the formation of oxygen radicals followed by lipid peroxidation may be the basis for the cardiotoxic effects of these drugs.

Daunomycin and adriamycin, two closely related anthracycline antibiotics, are active against a wide range of both animal (1,2) and human (3) neoplasms. Their clinical use is limited primarily by a dose-dependent cardiomyopathy (4). Electron microscopic examination of damaged hearts (4) shows disrupted plasma membranes, swollen mitochondria with disorganized cristae, and damaged myofibrils. The mechanism by which these cellular alterations are induced is unknown.

Both drugs contain quinone structures. Thus, these agents may be enzymatically reduced and may subsequently autoxidize, resulting in the generation of oxygen radicals and hydrogen peroxide (5,6). This potential for redox cycling has not generally been recognized in the metabolism of these anthracyclines. Their major metabolites are thought to be the products of reduction of the C-13 carbonyl carbon by a cytoplasmic aldo-keto reductase (7). In addition, aglycones may be formed through hydrolytic and reductive cleavage by microsomal enzymes (8). The aglycone may be O-demethylated and conjugated with either sulfate or glucuronate (9).

It is of interest that both daunomycin and adriamycin have been reported

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to initiate sulfite oxidation during incubation with rat liver microsomes (10). This finding supports the concept that these drugs may undergo cyclic reduction and autoxidation generating oxygen radicals. Such free radicals may initiate a peroxidation of endogenous lipids and cause membrane damage (11). We note in this connection that Myers, et al. (12) have recently reported that vitamin E, a free radical scavenger, decreases the toxicity of adriamycin in mice, presumably by ameliorating cardiac damage.

For these reasons, we have investigated the capacity of these drugs to participate in both redox cycling and oxygen radical formation which lead to the initiation of lipid peroxidation.

Materials and Methods: Liver microsomes were prepared from Sprague-Dawley rats after overnight fasting (13). Oxygen consumption was determined in a Gilson oxygraph fitted with a Clark-type electrode. A 20 mM Tris-HCl buffer, pH 7.4, was utilized in all experiments. The reaction volumes were 1.0 to 1.1 ml and the temperature was maintained at 37° C. The peroxidation of microsomal lipids was measured by the thiobarbituric acid (TBA) assay (14).

Purified P450 reductase (320 units/ml) was a generous gift from Drs. Rhea Craig and Wayne Bidlack. Superoxide dismutase (SOD) and catalase were obtained from the Sigma Chemical Company (St. Louis, Mo.); butylated hydroxyanisole (BHA) was obtained from the Nutritional Biochemicals Corporation (Cleveland, Ohio). Adriamycin-HCl and daunomycin-HCl were gifts from Dr. Kenneth Chan.

Although the results of single experiments are presented in the Figures and Tables, each experiment was repeated several times with essentially the same results.

Results: Fig. 1 shows the oxygen consumed by 10 nmoles of adriamycin in the presence of purified P450 reductase and NADPH. In the absence of either the drug or the enzyme, the amounts of oxygen consumed were negligible. Although not shown, substitution of NADH for NADPH produced no significant oxidation. However, it may be seen that with NADPH, the total amount of oxygen taken up was far in excess of that to be expected by simple stochiometric oxidation of the drug. When catalase was added during the incubation, oxygen was returned to the medium (ca. 20 nmoles) and the subsequent rate of oxygen utilization was approximately halved (8.5 nmoles O_2/min. prior to, and 4.0 nmoles O_2/min. subsequent to the addition of catalase). In other experiments, if catalase was not added, oxygen utilization continued until all of it was utilized (ca. 250

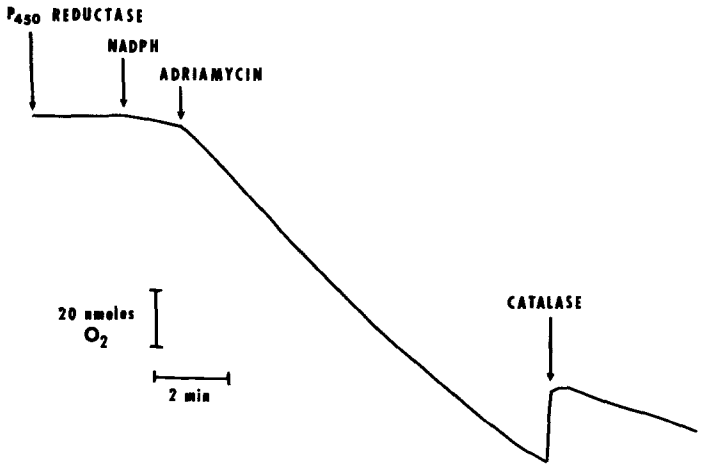

Figure 1: Oxygen consumption with 10 nmoles adriamycin. Where indicated, 16 units of P450 reductase, 0.8 μmoles NADPH, 10 nmoles adriamycin, and 15 units catalase were added to 1.0 ml buffer. See Materials and Methods for details.

nmoles). The substitution of daunomycin for adriamycin produced similar results.

The consumption of oxygen described above is accompanied by the formation of radicals capable of initiating the oxidation of sulfite. This is illustrated in Fig. 2 for experiments carried out with daunomycin, although similar results were obtained with adriamycin. In the presence of NADPH and P450 reductase, daunomycin (40 μM) consumed oxygen at an initial rate of 12.8 nmoles/min. (Fig. 2-A). When sulfite was included in the incubation medium, the rate increased to 75 nmoles/min. (Fig. 2-B). In the absence of the drug, there was no sulfite-dependent oxygen uptake. Fig. 3-C shows the partial inhibition of sulfite oxidation observed after addition of superoxide dismutase. This effect suggests that the superoxide anion (O_2^-) is generated during the course of the reaction and is the source of the hydrogen peroxide detected by the addition of catalase (Fig. 1). Although not shown in the illustrations, butylated hydroxyanisole had a small inhibitory effect on oxygen consumption (ca. 30%). Mannitol, an OH radical scavenger, had no measurable effect on sulfite oxidation.

Table 1 shows the initial rate of O_2 consumption and the total amount of

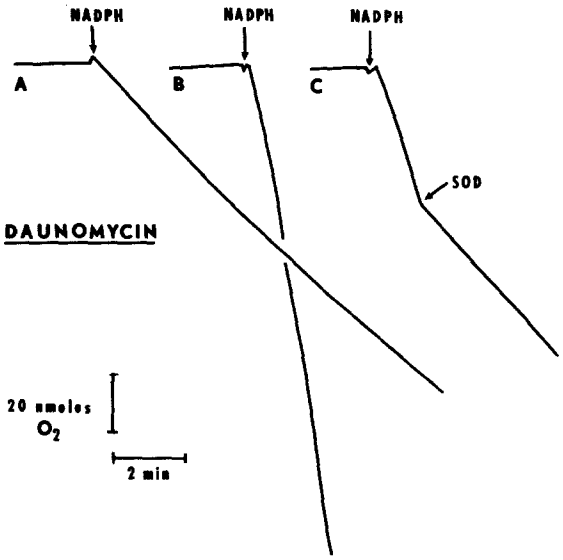

Figure 2: Initiation of sulfite oxidation with daunomycin. The complete system contained 6 units of P450 reductase, 2 μmoles EDTA and 40 μmoles of daunomycin in the standard buffer. The reactions were started by the addition of 20 μmoles NADPH. Curve A, sulfite was absent; Curve B, 20 μmoles Na_2SO_3 was present; Curve C, 20 μmoles Na_2SO_3 was present and 15 units of superoxide dismutase (SOD) were added at the time indicated.

Table 1: Daunomycin-stimulated oxygen consumption in rat liver microsomes.[1]

Additions	O_2 Consumption	
	Initial Rate (30 sec.) nmoles/min.	Total O_2 (10 min.) nmoles
NADPH (500 nmoles)	23	166
NADPH + daunomycin (50 nmoles)	40	250[2]
NADPH + daunomycin + butylated hydroxyanisole (10 μg)	29	178
NADPH + butylated hydroxyanisole	20	137
NADH (500 nmoles)	14	83
NADH + daunomycin (50 nmoles)	18	160
NADH + daunomycin + butylated hydroxyanisole (10 μg)	11	63
NADH + BHA	13	70

[1] Total microsomal protein/tube = 1.75 mg

[2] Total amount of O_2 consumed after 5.2 minutes

O_2 consumed during a ten-minute incubation with rat liver microsomes rather than with P450 reductase. It may be seen that in the presence of daunomycin, the initial rate of O_2 uptake was almost twice that observed in its absence and that after only 5.2 minutes, all of the O_2 (250 nmoles) had been consumed. In the absence of daunomycin, a total of 166 nmoles were consumed during the ten minute period. Butylated hydroxyanisole, while having only a small effect on O_2 uptake in the absence of daunomycin, almost completely prevents the stimulation observed in the presence of the drug. It may also be seen in Table 1 that, with microsomes, the substitution of NADH for NADPH yielded effective drug oxidation. However, with NADH, the initial rate of oxygen uptake and the total amount of oxygen consumed were diminished.

Table 2 shows the result of a typical experiment in which lipid peroxidation in the microsomal preparation was measured by determining the amount of thiobarbituric acid-reactive material formed during incubations of ten and twenty minutes. It may be seen in the table that both daunomycin and adriamycin gave rise to lipid peroxides in the presence of NADPH. However, parallel to the experiments on oxygen consumption (Table 1) the capacity to produce lipid peroxides was markedly diminished when NADH was substituted for NADPH.

Discussion: The consumption of oxygen many times in excess of the amount of drug present, coupled with the dependence on a source of reducing equivalents observed in the above experiments, cannot be explained by the known metabolism of these drugs. These phenomena are highly suggestive of a redox cycling. Other quinoid compounds are known to undergo a similar reduction and autoxidation (5,6). The addition of catalase (Fig. 1) resulted in a return of oxygen to the medium, suggesting that hydrogen peroxide was generated. After this addition, the rate of oxygen uptake was about half the prior rate. This decrease would be expected if peroxide were continually generated by the autoxidation of the reduced drug. About half of the oxygen would be returned as molecular oxygen by catalase.

The initiation of sulfite oxidation by daunomycin has been previously ob-

Table 2: Effects of daunomycin and adriamycin on lipid peroxidation in rat liver microsomes.

Additions	Absorbance at 532 nm	
	10 min.	20 min.
NADPH (500 nmoles)	0.110	0.255
NADPH + Daunomycin (50 nmoles)	0.260	0.466
NADPH + Adriamycin (50 nmoles)	0.181	0.413
NADH (500 nmoles)	0.038	0.049
NADH + Daunomycin (50 nmoles)	0.071	0.123
NADH + Adriamycin (50 nmoles)	0.045	0.089

Values observed at zero time were subtracted. The values reported are the average of duplicates, which agreed within 10% of each other, in a typical experiment.

served (10), but not with a purified enzyme. That sulfite oxidation can be inhibited by superoxide dismutase (SOD) (Fig. 2) indicates that the superoxide radical is indeed generated by the redox cycling of the drugs and is a likely intermediate in the formation of hydrogen peroxide. The lack of inhibition by mannitol suggests that hydroxy radicals are not involved in the autoxidation of the drugs and initiation of sulfite oxidation.

In the experiments with the purified P450 reductase, NADH was, as to be expected, not active in the generation of radicals. However, the results shown in Table 2 demonstrate that intact microsomes are able to utilize NADH as well as NADPH in the redox cycling of these anthracyclines. Clearly, microsomal enzymes other than P450 reductase may participate in the metabolism which leads to free-radical formation. The inhibitory effect of butylated hydroxyanisole (BHA) on microsomal oxygen utilization (Table 2) suggests that this oxygen consumption was, in part, the consequence of a peroxidation of endogenous lipids.

It is known that superoxide dismutase levels in the heart are lower than in many well-perfused organs such as liver and kidney (15). Lipid peroxidation initiated by free-radicals generated through the redox cycling of daunomycin and adriamycin suggested in these experiments could well be the basis for the cardiotoxicity of these agents and the possibility of preventing this serious

side effect by either prior or simultaneous treatment with antioxidants would be an attractive one.

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