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INHIBITION BY ADRIAMYCIN OF A METMYOGLOBIN REDUCTASE FROM BEEF HEART

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The effectiveness of the anti-tumor agent adriamycin is limited by a dose-related cardiotoxicity which has been shown to develop in both animals and humans. The mechanism by which the drug causes congestive heart failure is not certain. However, it has been suggested that it may be related to the ability of adriamycin and its parent compound daunomycin to autoxidize, form radicals, and thereby cause peroxidative damage to membranes and other cellular constituents (1,2). In the course of isolating and characterizing a previously undescribed metmyoglobin (MMb) reductase from beef heart, we have found that adriamycin is able to non-competitively inhibit the NADH- and NADPH-dependent activities of this enzyme. The results presented in this report lead us to suggest that at least part of the cardiotoxicity of adriamycin may be attributed to its ability to alter the state of myoglobin reduction in cardiac tissue.

Materials and Methods. The details of the isolation and characterization of the beef heart MMb reductase will be presented elsewhere, but a brief description of the isolation procedure is given here. Fresh beef hearts obtained at the time of slaughter were washed thoroughly with cold water. After removal of fat and atria, the tissue was cut into small pieces and washed with many changes of ice-cold 10 mM potassium phosphate buffer, pH 7.0, to remove as much hemoglobin as possible. The tissue was frozen in the buffer at -20° and thawed as needed. Thawed tissue was again washed with several changes of buffer. All steps were carried out at 0-4° unless otherwise indicated. Pieces of heart were homogenized in a Waring blender as a 1:5 (w/v) homogenate in buffer. The homogenate was centrifuged at 100,000 g for 30 min and the lipid layer discarded. The resulting clear, pink supernatant solution was subjected to precipitation by ammonium sulfate at room temperature. The fraction precipitating between 70 and 80% saturation was collected, dissolved in 5 mM Tris-1 mM EDTA, pH 8.3, and dialyzed extensively against this buffer in an Amicon Ultrafiltration apparatus using a PM 30 filter. It was placed on a DEAE-cellulose column in the Tris-EDTA buffer and eluted with the same buffer. The hemoglobin remained on the column while the enzyme eluted in the void volume. The fraction eluting in Tris-EDTA was concentrated and dialyzed into 20 mM potassium phosphate buffer, pH 6.5, containing 5% (v/v) glycerol and 1 mM EDTA. The resulting solution was placed on a CM cellulose column to which a linear gradient of the starting buffer and the same buffer containing 250 mM NaCl was applied. The enzyme eluted as a single major peak of activity of about 200 mM NaCl. It was not homogeneous but contained several proteins as judged by the results of SDS polyacrylamide gel electrophoresis.

Assays for enzyme activity were carried out in 10 mM potassium phsophate buffer, pH 7.0, in a 1.0 ml reaction volume at 37 °. Reduced nucleotide concentration was 0.2 mM unless otherwise specified. MMb reductase activity was measured with 0.05 mM MMb and 2.5 μM methylene blue in the reaction mixture. Appearance of oxymyoglobin was followed at 582 nm and an extinction coefficient of 14.4 mM^{-1} was used (3). Reduction of 2,6-dichlorophenolindophenol (DCIP) at a final concentration of 20 μM was followed at 600 nm using an

extinction coefficient of 21 mM^{-1} (4). In all cases, blanks containing all components except enzyme were also run.

The effect of O_2 on the reaction was determined by assaying enzyme activity in an anaerobic cuvette. After thoroughly flushing with N_2, the reaction was begun with the addition of enzyme from the side arm of the closed cuvettes.

Oxymyoglobin was prepared from MMb by incubating 1 mM in 10 mM potassium phosphate buffer, pH 7.0, at 37° with 1 mM NADPH and 12.5 μM methylene blue for 1 hr. The solution was dialzyed overnight against the buffer to remove the nucleotide and dye. After removing the precipitate by centrifugation, the reduction of the MMb to oxymyoglobin was confirmed by spectral analysis. Approximately 50 per cent of the MMb was converted to oxymyoglobin by this procedure. Protein concentration was determined by the method of Lowry et al. (5). Horse heart myoglobin from Sigma Chemical Co., St. Louis, MO, was found by spectral analysis to be essentially in the met form and was used without further purification. Adriamycin was a gift from Soc. Farmaceutici Italia, Milan, Italy. The data shown are from single experiments which have been repeated several times.

Results. The capacity of the enzyme to reduce MMb in the presence of methylene blue compared with its ability to use DCIP as a terminal electron acceptor is shown in Table 1. As with methemoglobin reductases from red cells (6), reduction of DCIP is much faster than reduction of the heme protein. In the absence of methylene blue, the enzyme can only reduce MMb at about 2 per cent of the rate shown. This is also qualitatively similar to the methemoglobin redutases of the red cell which are much more efficient in the presence of an intermediate electron carrier such as methylene blue or cytochrome b_5 (7-9). The activity of the two acceptors differed slightly with respect to the two nucleotides; when MMb was used as the acceptor, NADH was somewhat preferred as the substrate, whereas NADPH was preferred when DCIP served as the acceptor. However, 20 μM adriamycin inhibited both NADH-dependent reactions by nearly 50 per cent, while NADPH-dependent reactions were depressed by 30-35 per cent. It can be seen in Table 1 that the ability of the drug to inhibit the enzyme is not dependent on O_2. In the absence of O_2, the enzyme activity was lower, but 20 μM adriamycin inhibited both nucleotide-dependent activities to an even greater extent than in the presence of O_2. It is not clear why the absence of oxygen depressed the enzyme activity.

The kinetics of the enzyme reaction for NADH and NADPH in the presence and absence of 20 μM adriamycin are shown in Fig. 1. The K_m of the reaction for NADH is 125 μM and for NADPH is 59 μM. It can be seen that the drug is a non-competitive inhibitor for both the reduced nucleotides, the K_i in the presence of NADH being 28 μM and for NADPH 38 μM.

We have also found that adriamycin is able to enhance the oxidation of oxymyoglobin in the absence of enzymes. Thus, in the presence of 40 nmoles of drug, oxymyoglobin was oxidized at a rate of 40 pmoles/min, whereas, in the absence of the drug, the rate of autoxidation was only 12 pmoles/min.

Table 1. Inhibition of enzyme activity by adriamycin

Drug concn	MMb reductase*		DCIP reductase*		DCIP reductase* (anaerobic)	
(μM)	NADH	NADPH	NADH	NADPH	NADH	NADPH
0	2.70	1.31	68.8	97.4	27.4	40.4
20	1.48 (45%)	0.90 (31%)	35.1 (49%)	62.9 (35%)	10.3 (62%)	23.4 (42%)
40	1.31 (51%)	0.74 (44%)	30.0 (56%)	56.2 (42%)	-	-

*Enzyme activities were measured as described in Materials and Methods and are expressed as nmoles substrate reduced/min/mg of protein. Figures in parentheses represent per cent inhibition of activities without adriamycin. To investigate the effect of O_2, anaerobic cuvettes were flushed with N_2 and the reaction was started with enzyme solution contained in the side arm.

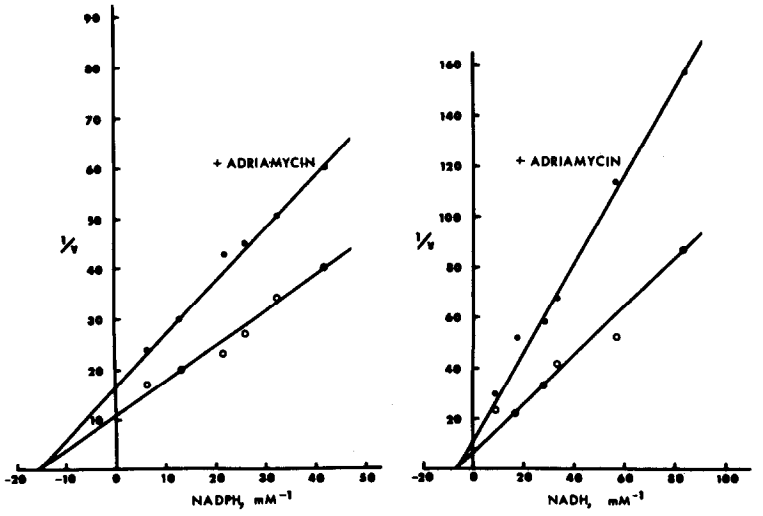

Fig. 1. Lineweaver-Burk plot showing non-competitive inhibition of DCIP reductase activity by 20 μM adriamycin. Enzyme activities were determined as described in Materials and Methods using the concentrations of reduced nucleotides indicated in the figure. Velocity is in $(\text{nmoles/min/mg})^{-1} \times 10^3$.

Discussion. The mechanism by which adriamycin causes damage to heart muscle is of interest because of its wide use as an anti-tumor agent. The cumulative dose that can be administered is limited by the drug's cardiotoxic side effects (10). A number of adriamycin-induced-biochemical effects have been shown in whole animals, cells and subcellular preparations. Electron transport in isolated mitochondria is inhibited (11), and cell surface properties are

changed after incubation with the drug (12). Levels of reduced glutathione and of the enzyme glutathione peroxidase are lowered in treated animals (13,14). The latter effects would tend to increase the peroxidative damage in adriamycin-containing cells, since it has been shown that the compound can cycle through its reduced and oxidized forms in an enzyme-dependent process using NADPH and O_2, thereby generating radicals (1,2).

In this paper, we have shown that adriamycin interferes with a soluble bovine heart enzyme that is able to reduce MMb to its oxygen-carrying form. Additionally, the drug accelerates the oxidation of oxymyoglobin to MMb. It is of interest that the interaction of adriamycin with red cells causes radical formation which is dependent on the presence of oxyhemoglobin (15,16). The similarity in the physical and chemical properties of hemoglobin and myoglobin suggests that an analogous reaction may occur between the drug and oxymyoglobin. Such a reaction would result in an increase in peroxidative damage to cellular components.

We visualize that in the presence of adriamycin, not only would the formation of MMb be accelerated but the enzymatic mechanism employed to reduce MMb would be inhibited, resulting in a decrease in intracellular oxygen-carrying capacity in the heart. That myoglobin plays an important role in providing oxygen to muscle has been demonstrated (17). Although skeletal muscle also contains myoglobin, adriamycin concentrations do not reach the levels that are achieved in cardiac muscle (18,19).

The results presented here suggest that adriamycin has the potential to exert at least part of its cardiotoxic effect by influencing myoglobin oxidation and reduction.

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