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ADRIAMYCIN-INDUCED OXIDATION OF MYOGLOBIN[1]

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SUMMARY: Numerous mechanisms have been invoked to explain the cardiotoxicity of Adriamycin, most of which share a requirement for iron. Adriamycin is chemically reactive with iron loosely associated with subcellular membranes as well as with ferritin and the heme iron of hemoglobin. The present investigation examined whether Adriamycin also reacts with myoglobin, an abundant source of iron in cardiac muscle. Adriamycin caused a 4-fold stimulation of the autoxidation of oxymyoglobin to metmyoglobin. Hydrogen peroxide is an obligatory intermediate as catalase completely inhibited the reaction. Superoxide dismutase, however, was without effect. This interaction of Adriamycin with myoglobin may impose significant restrictions on oxygen storage and delivery in vivo. In light of the abundance of myoglobin and the deficiency of catalase in the heart, this interaction with myoglobin may be an important determinant of the cardioselective toxicity of Adriamycin.

Adriamycin (Doxorubicin) is an anthracycline antibiotic effective in the treatment of a number of neoplastic diseases but with limited clinical utility due to the high incidence of cardiomyopathy (1). The futile redox cycling of Adriamycin has been invoked in each of the various cytopathological responses caused by the drug. These include the scission and condensation of DNA, peroxidation of phospholipid membranes, depletion of cellular reducing equivalents, interference with mitochondrial respiration, and disruption of cell calcium homeostasis (2). Oxygen free radicals are implicated in each of these responses and there exists a common requirement for iron, which is particularly evident for Adriamycin-induced lipid peroxidation (3,4). With few exceptions, metal chelators are potent inhibitors of Adriamycin-induced oxidative damage. The mechanism of protection may be either interference with the iron-catalyzed generation of hydroxyl free radicals or prevention of the formation of electrochemically reactive coordination complexes between the metal and Adriamycin (5-7).

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Abbreviations used: Adria, Adriamycin; $MbFe^{2+}$-O_2, oxymyoglobin; $MbFe^{2+}$, deoxymyoglobin; $MbFe^{3+}$, metmyoglobin; $MbFe^{4+}$, ferrylmyoglobin; SOD, superoxide dismutase.

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The physiological source of iron in vivo has not been established. Adriamycin causes the reductive release of iron from subcellular membranes and ferritin (8-10). Adriamycin also alters the redox state of the heme-iron of hemoglobin (11). Although these metaloproteins may interact with iron *in vitro*, they fail to explain the selective toxicity of Adriamycin to cardiac tissue. In light of the preferential distribution of myoglobin in muscle, an oxidative interaction of Adriamycin with this heme protein, similar to that observed with hemoglobin (11), may be invoked to explain the cardioselective target organ toxicity of the drug. This is especially relevant since oxygen free radicals are known to participate in electron transfer reactions with the heme iron of myoglobin (12-14). Furthermore, damage resulting from such a free radical-generating process would be compounded by the lack of antioxidant enzymes in the heart compared with other tissues (15,16).

MATERIALS AND METHODS

Reagents - Metmyoglobin from horse skeletal muscle (95-100%), catalase (EC 1.11.1.6), xanthine oxidase (EC 1.2.3.2), glucose oxidase (EC 1.1.3.4), and peroxidase (EC 1.11.1.7) were purchased from Sigma Chemical Co. (St. Louis, MO). Cu/Zn-Superoxide dismutase (SOD; EC 1.15.1.1; 3000 u/mg protein) was obtained from the Biotics Research Corporation (Houston, TX). All reagents were dissolved in 10 mM HEPES (pH 7.4). Adventitious metal ions were removed from the buffer by column chromatography over Chelex-100 (Bio Rad Laboratories). Adriamycin was purchased from Adria Laboratories (Columbus, OH).

Stock solutions of metmyoglobin were prepared by Sephadex G-25 chromatography (8) and adjusted to pH 7.4. The concentration of metmyoglobin was estimated using a molar extinction coefficient of $E_{630nm}4.0mM^{-1}cm^{-1}$ (17). Oxymyoglobin was prepared by reduction of metmyoglobin with excess sodium dithionite (18). Unreacted dithionite was removed by Sephadex G-25 chromatography and the oxymyoglobin fraction brought to pH 7.4. The concentration of oxymyoglobin was estimated spectrophotometrically using an extinction coefficient of $E_{582nm}14.4mM^{-1}cm^{-1}$ (19). The criterion for completeness of the reduction procedure was $A_{582nm}/A_{543nm} \geq 1.0$, otherwise the preparation was discarded.

Stock solutions of catalase, xanthine oxidase and glucose oxidase were purified by chromatography on Sephadex G-25 (8). Each enzyme fraction was collected and the pH adjusted to 7.4. Catalase activity was estimated from the rate of disappearance of hydrogen peroxide (20) using an extinction coefficient of $E_{240nm}43.6mM^{-1}cm^{-1}$ (21). The activity of xanthine oxidase was determined from the rate of formation of uric acid from xanthine (0.8 mM), measured spectrophotometrically using an extinction coefficient of $E_{293nm}12.5\ M^{-1}cm^{-1}$ (22). Glucose oxidase activity was estimated according to the horseradish peroxidase coupled oxidation of *o*-dianisidine (23).

Methods - Oxidation of oxymyoglobin - Reactions containing 25 μM oxymyoglobin in 1ml of 10 mM HEPES (pH 7.4) were incubated in room air at 25°C in the presence or absence of one or more of the following: Adriamycin (50 μM), catalase (350 units/ml), and SOD (8 μg protein/ml). The rate of oxidation of oxymyoglobin was determined by monitoring the absorbance at 582 nm for 10 h. Changes in the absorbance spectrum were recorded at time zero and 10 h of incubation.

Anaerobic, xanthine/xanthine oxidase-catalyzed reduction of metmyoglobin - Reactions containing metmyoglobin (25 μM), xanthine oxidase (0.01 units/ml), SOD (8 μg protein/ml), and catalase (350 units/ml) were incubated anaerobically with or without Adriamycin (50 μM) and/or xanthine (100 μM) in 1 ml of 10 mM HEPES (pH 7.4) for 30 min at 25°C. To conduct the above incubations anaerobically, solutions were bubbled with argon for 5 min prior to the addition of xanthine oxidase after which they were immediately sealed with parafilm. Glucose (5 mM) and glucose oxidase (10 units/ml) were included to insure complete anaerobicity during the course of the incubation. Catalase was added to prevent glucose oxidase generated hydrogen peroxide from interfering with the reaction. The rate of metmyoglobin reduction was determined by monitoring the absorbance at 560 nm. Changes in the absorbance spectra were recorded at time-zero and 30 min of incubation. Because the absorption spectra of deoxymyoglobin and ferrylmyoglobin are similar, formation of deoxymyoglobin was confirmed by bubbling the final 30 min reaction mixture with oxygen for 5 min and recording the resulting absorbance spectrum.

Statistics - Data were analyzed by analysis of variance and the difference between paired comparisons was considered to be statistically significant using the least significant difference test ($p<0.05$).

RESULTS

Reduction of Metmyoglobin - Although Adriamycin did not enhance the oxidation of metmyoglobin (data not presented), the anaerobic reduction of metmyoglobin by xanthine oxidase was dependent on the drug (Fig. 1 and Table 1). Samples incubated in the absence of either Adriamycin or xanthine showed a small time-dependent decrease in absorbance at 560 nm, presumably resulting from the oxidation of contaminating oxymyoglobin.

After 30 min of incubation, there was a substantial increase in absorbance in the range of 525 nm to 600 nm and decreases in absorption centered at 630 nm and 500 nm (Fig. 2), consistent with the disappearance of metmyoglobin and formation of deoxymyoglobin. The formation of deoxymyoglobin was confirmed by bubbling the sample with oxygen and re-recording the absorbance spectrum. The resulting spectrum had absorption maxima at approximately 540 nm and 580 nm (Fig. 2), corresponding with the known absorbance spectrum of oxymyoglobin.

Oxidation of Oxymyoglobin - The aerobic autoxidation of oxymyoglobin during a 10 h incubation is depicted in figure 3 as the decrease in absorbance at 582 nm, a maximum for oxymyoglobin. Including Adriamycin in the reaction mixture resulted in nearly a 4-fold stimulation of the rate of oxymyoglobin oxidation (Fig. 3; Table 2). The addition of SOD had no effect. Catalase, however, inhibited the stimulation caused by Adriamycin, the result being a rate of oxidation only slightly greater than that of the control.

Spectral interference by Adriamycin made positive identification of the oxidation state of myoglobin difficult. To provide additional evidence for the identity of the hemeproteins, a reaction containing Adriamycin, SOD, and oxymyoglobin was prepared. At time-zero, an aliquot of the

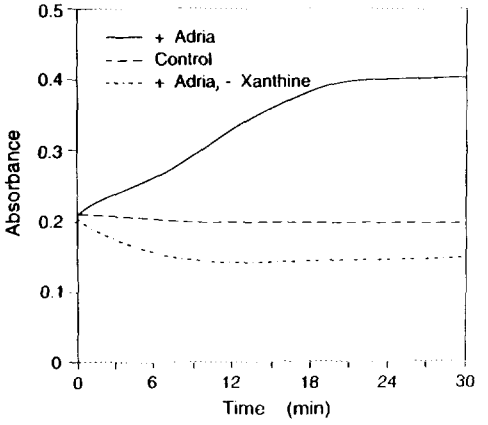


Figure 1. *Adriamycin-Dependent Reduction of Metmyoglobin to Deoxymyoglobin.* Controls initially contained xanthine/xanthine oxidase and metmyoglobin (25 μM) in 10 mM HEPES (pH 7.4). Reactions were conducted anaerobically in the presence of glucose/glucose oxidase, SOD and catalase. The addition of 50 μM Adriamycin (Adria) or the deletion of xanthine was as indicated. Results are presented as the absorbance at 560 nm (maximum for deoxymyoglobin) during the 30 min incubation and are representative of those obtained on several occasions.

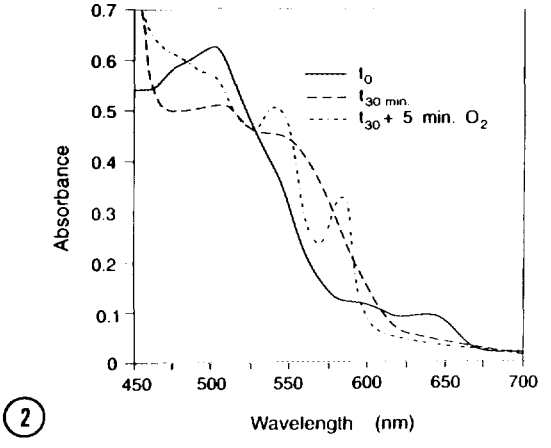
Table 1

Adriamycin-Dependent Anaerobic Reduction of Metmyoglobin

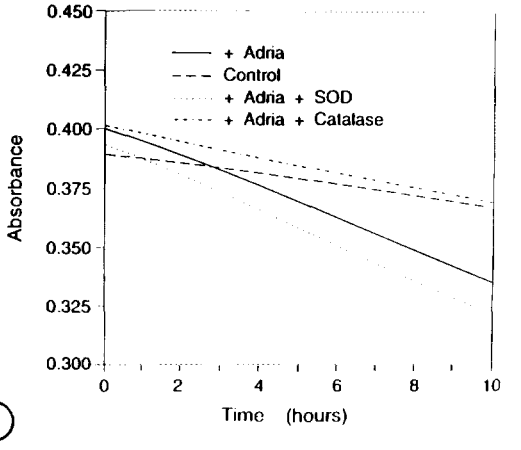
	ΔA_{560}/min
Control	-0.0059±.0028
+ Adria (50 μM)	0.0072±.0002 *
+ Adria, - xanthine	-0.0012±.0002

Controls contained xanthine/xanthine oxidase, glucose/glucose oxidase, SOD, catalase and metmyoglobin (25 μM) in 10 mM HEPES (pH 7.4). Additions of Adriamycin (Adria) were as noted. Maintenance of anaerobicity was as stated in the methods. Results are presented as the initial rate of formation of deoxymyoglobin as measured by the absorbance change at 560 nm. Results are the mean±SE for three separate experiments. The asterisk (*) indicates a statistically significant difference compared to control ($p<0.05$).

sample was chromatographed on Sephadex G-25, the myoglobin fraction collected, and the absorbance spectrum recorded. This same procedure was then repeated after 10 h of incubation. The spectra are presented in figure 4. Although quantitative comparisons cannot be made due to the incomplete recovery of the heme proteins, it is evident that a significant change occurred in the absorbance spectrum of oxymyoglobin during the 10 h incubation. The diminished maxima at



(2)



(3)

Figure 2. *Absorption Spectra for Adriamycin-Induced Reduction of Metmyoglobin.* Shown is the absorbance spectrum of the sample containing xanthine/xanthine oxidase, Adriamycin (50 μM), and metmyoglobin (25 μM) in 10 mM HEPES (pH 7.4) and incubated for 0 or 30 min. Also presented is the absorbance spectrum for the 30 min sample following oxygenation. The reaction was conducted anaerobically as outlined in the Materials and Methods section. The tracings are typical of those obtained on several occasions.

Figure 3. *Oxidation of Oxymyoglobin; Disappearance of Oxymyoglobin.* Controls initially contained oxymyoglobin (25 μM) in 10 mM HEPES (pH 7.4). Additions of 50 μM Adriamycin (Adria), SOD (8 μg protein/ml), and catalase (350 units/ml) were as indicated. Results are presented as the absorbance at 582 nm (maximum for oxymyoglobin) during the 10 h incubation. Results are representative of those obtained on several occasions.

Table 2

Adriamycin-Catalyzed Oxidation of Oxymyoglobin

	ΔA_{582}/hr
Control	-0.00175±.00013
+ Adria (50 μM)	-0.00673±.00032 *
+ Adria + SOD (8 μg protein/ml)	-0.00754±.00014 *
+ Adriamycin + Catalase (350 units/ml)	-0.00288±.00018

Controls contained oxymyoglobin (25 μM) in 10 mM HEPES buffer (pH 7.4). Additions of Adriamycin (Adria), SOD, and catalase were as indicated. Results are presented as the rate of disappearance of oxymyoglobin as measured by the absorbance change at 582 nm. Results are the mean±SE of three experiments. The asterisk (*) indicates a statistically significant difference compared to the control sample ($p<0.05$).

540 nm and 580 nm and the increase in absorbance at 500 nm and 630 nm are consistent with the oxidation of oxymyoglobin to metmyoglobin.

Iron Release - Attempts to measure iron-release from myoglobin were unsuccessful. We observed no indication that Adriamycin stimulates iron release from myoglobin under any of the conditions examined.

DISCUSSION

The interaction between iron-centered heme proteins and hydrogen peroxide or oxygen free radicals is well-documented and implicated in a number of cytopathic reactions (12,24,25). The

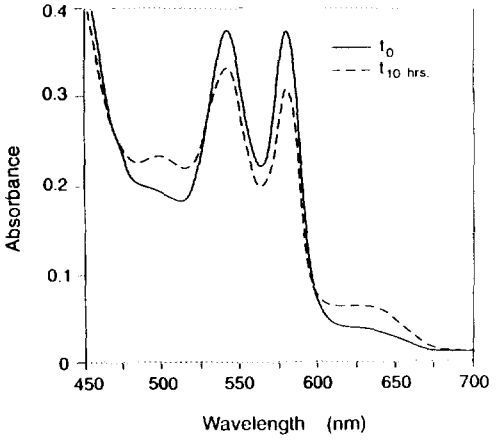


Figure 4. *Oxidation of Oxymyoglobin; Isolation of Myoglobin.* The absorbance spectrum of the myoglobin fraction collected after chromatography on Sephadex G-25 is illustrated. Samples initially contained SOD (8 μg protein/ml), Adriamycin (50 μM), and oxymyoglobin (25 μM). The myoglobin fraction was isolated at time zero and 10 h and the absorbance spectra recorded.

present investigation provides the first demonstration that Adriamycin interacts directly with the heme-iron of myoglobin, which may be a significant factor in the cardiotoxicity of the drug.

Reduction of Metmyoglobin

Under anaerobic conditions, xanthine oxidase reduces Adriamycin to the semiquinone free radical [reaction 1] (26,27). Aerobically, dioxygen is the preferred electron acceptor and no semiquinone free radical of Adriamycin is detectable. The evidence presented suggests that the semiquinone of Adriamycin reduces metmyoglobin to deoxymyoglobin [reaction 2], similar to the reaction of Adriamycin with methemoglobin and ferricytochrome c (11).

$$\text{Adr} \xrightarrow{\text{X/XO}} \text{Adr}^{\bullet} \qquad [1]$$

$$\text{Adr}^{\bullet} + \text{MbFe}^{3+} \longrightarrow \text{Adr} + \text{MbFe}^{2+} \qquad [2]$$

$$\text{MbFe}^{2+} \xrightarrow{O_2} \text{MbFe}^{2+}\text{-}O_2 \qquad [3]$$

Bubbling the reaction mixtures with oxygen was performed to confirm that the product of the anaerobic reduction of metmyoglobin was in fact deoxymyoglobin [reaction 3]. A product with an absorbance spectrum identical to that of oxymyoglobin was observed (Fig. 2). As additional evidence, the myoglobin fraction of selected samples was isolated by Sephadex G-25 chromatography and the spectra was clearly that of oxymyoglobin (data not shown).

Oxidation of Oxymyoglobin

Autoxidation of oxymyoglobin in vivo is slow and not likely to elicit significant oxidative damage. In the presence of Adriamycin, however, the rate of oxidation of oxymyoglobin is increased almost four-fold which may be sufficient to overwhelm the finite cellular defense mechanisms of the heart. There are several possible mechanisms to account for the stimulation of oxymyoglobin oxidation by Adriamycin. Various anions (N_3^-, SCN^-, OCN^-, F^- and Cl^-) stimulate the displacement of $O_2^{\bullet}$ from hemeproteins via an S_N2-type nucleophilic attack (28,29). Because of steric hindrance, however, it is unlikely that Adriamycin is able to gain access to the heme pocket, an assertion supported by the fact that formation of a myoglobin-Adriamycin complex was not observed.

Wallace *et al.* (30,31) suggest that the hydrophobic nature of the heme pocket functions to exclude polar anions from binding to the heme-iron. The presence of a strong nucleophile in the vicinity of the heme-iron provides sufficient labilization of the iron valence electron to permit oxidation at a site remote from the heme-iron. This may be accomplished by altering the conformation of the globin moiety, thereby allowing a one-electron transfer from the heme-iron to the nucleophile with loss of the bound oxygen [reaction 4]. The products are metmyoglobin, oxygen, and the semiquinone free radical of Adriamycin ($Adr^{\bullet}$). A similar reaction mechanism has been proposed for both menadione and acetylphenylhydrazine-stimulated oxidation of oxyhemoglobin (32,33).

$$\text{MbFe}^{2+}\text{-}O_2 + \text{Adr} \longrightarrow \text{MbFe}^{3+} + O_2 + \text{Adr}^{\bullet} \qquad [4]$$

$$\text{Adr}^{\bullet} + \text{MbFe}^{2+}\text{-}O_2 \longrightarrow \text{MbFe}^{3+} + H_2O_2 + \text{Adr} \qquad [5]$$

The semiquinone radical of Adriamycin formed during the oxidation of oxymyoglobin may be involved in a number of subsequent reactions. The semiquinone free radical may participate in the 2-electron reduction of the heme-bound oxygen of oxymyoglobin to form hydrogen peroxide, the parent quinone, and metmyoglobin [reaction 5]. In this reaction, oxygen accepts one electron from the ferrous heme-iron and one from the Adriamycin semiquinone radical. This type of reaction is proposed to occur during the oxidation of oxyhemoglobin and oxymyoglobin by diphenol, catechol, and *p*-hydroquinone (34). The Adriamycin semiquinone free radical may also reduce molecular oxygen to superoxide (32,33). We failed, however, to detect any stimulation of oxygen consumption or superoxide anion radical generation by Adriamycin indicating that oxymyoglobin may be preferred over dioxygen as the electron acceptor for the Adriamycin semiquinone free radical.

Catalase was a potent inhibitor of the Adriamycin-induced oxidation of oxymyoglobin indicating that hydrogen peroxide is a primary oxidant in the reaction. The 2-electron oxidation of oxymyoglobin by hydrogen peroxide [reaction 6] is suggested to be more favorable than the reaction of hydrogen peroxide with metmyoglobin (34,35). The ferrylmyoglobin ($MbFe^{4+}$) formed likely undergoes rapid reduction to metmyoglobin concomitant with the oxidation of one molecule of oxymyoglobin to metmyoglobin [reaction 7]. Accordingly, the reduction of hydrogen peroxide to water results in the oxidation of two molecules of oxymyoglobin.

$$MbFe^{2+}\text{-}O_2 + H_2O_2 \longrightarrow MbFe^{4+} + O_2 \qquad [6]$$

$$MbFe^{4+} + MbFe^{2+}\text{-}O_2 \longrightarrow (2)\ MbFe^{3+} \qquad [7]$$

In conclusion, Adriamycin was found to be required for the anaerobic reduction of metmyoglobin by xanthine oxidase. In the presence of oxygen, Adriamycin also stimulates the spontaneous oxidation of oxymyoglobin to metmyoglobin, reducing the oxygen-carrying capacity of this important heme protein. Hydrogen peroxide is an important electron acceptor as catalase provides near complete protection against the stimulation of oxymyoglobin oxidation by Adriamycin. This may be of particular significance to the cardiac toxicity of Adriamycin since heart tissue contains virtually no catalase activity (15). In view of the abundance of myoglobin in cardiac tissue and the deficiency of catalase activity, this interaction of Adriamycin with myoglobin may have important implications with respect to the cardioselective toxicity of the drug.

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