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STIMULATION OF MYOGLOBIN-DEPENDENT LIPID PEROXIDATION BY ADRIAMYCIN[1]

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SUMMARY: The objective of this investigation was to determine whether myoglobin can serve as a physiological source of iron in the catalysis of Adriamycin-stimulated lipid peroxidation. In vitro peroxidation of arachidonic acid was stimulated 2-fold by 50 μM Adriamycin, the extent of lipid peroxidation being dependent on the duration of incubation and the concentrations of both Adriamycin and myoglobin. Differential inhibition studies suggest that hydroxyl radicals are involved in this reaction; however, superoxide and hydrogen peroxide were not implicated. The results demonstrate a potentially important stimulation by Adriamycin of myoglobin-dependent membrane-lipid peroxidation. In light of the abundance of myoglobin and the deficiency of oxygen radical detoxifying enzymes in the heart, this interaction may be a significant determinant of the cardioselective toxicity of Adriamycin.

Adriamycin (Doxorubicin) is a broad-spectrum antibiotic effective in the treatment of a number of neoplasms including leukemias, lymphomas and a variety of solid tumors. The clinical utility of Adriamycin is, however, limited by the high incidence of irreversible cardiomyopathy in patients receiving a cumulative dose exceeding 550 mg/m^2 (1). The prevailing hypothesis for the mechanism of Adriamycin cardiotoxicity involves futile redox cycling initiated by univalent reduction of Adriamycin to the highly reactive semiquinone free radical (2). Redox cycling of Adriamycin occurs in virtually all tissues generating assorted reactive oxygen species including, superoxide anion free radicals, hydrogen peroxide, and hydroxyl free radicals (3,4).

Oxygen free radical-dependent lipid peroxidation is implicated as the principal mechanism of Adriamycin cytotoxicity (4,5). Of particular interest is the requirement for iron (6,7).

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Abbreviations used: Adria, Adriamycin; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; Cat, catalase; benz, benzoate; mann, mannitol; dmu, dimethylurea; def, desferrioxamine; asc, ascorbate; BHT, butylated hydroxytoluene.

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Adriamycin-induced peroxidation of rat liver microsomal lipids is stimulated six-fold by ferric chloride (8,9). Additionally, metal chelators are potent inhibitors of Adriamycin-stimulated lipid peroxidation and the iron-chelating ICRF compounds are effective in limiting Adriamycin toxicity *in vivo* (10).

Adriamycin complexes with ferric iron and, by intramolecular rearrangement, reduces it to ferrous iron with the concomitant production of a one-electron oxidized drug radical (10-12). This radical can reduce oxygen or hydrogen peroxide to yield the hydroxyl free radical, a potent initiator of lipid peroxidation. Regarding the source of intracellular iron, Thomas and Aust (13) demonstrated that Adriamycin, in the presence of an enzymatic reducing system, catalyzes the superoxide anion free radical-dependent reductive release of iron from ferritin. Associated with this is the ferritin-dependent stimulation of lipid peroxidation by Adriamycin (14). While ferritin is a significant source of iron in the liver, its concentration in the heart is comparatively low. Furthermore, the reducing systems necessary for generating the semiquinone free radical are far more abundant in liver than in heart. Since hepatotoxicity is not the limiting clinical problem, Adriamycin-stimulated release of iron from ferritin may not be a significant consideration in the mechanism of cardiotoxicity.

A potential source of iron in cardiac tissue is myoglobin. Myoglobin is second only to hemoglobin in the quantity of iron stored in vivo, accounting for approximately ten percent of the total body iron store (15). Moreover, myoglobin is an efficient catalyst of lipid peroxidation, a process which involves redox cycling of the heme-iron (16-20). The abundance of myoglobin in the heart, and the potential reactivity of the heme-iron, may confer particular significance to this iron-containing protein as an important determinant of the cardioselective toxicity of Adriamycin.

In the preceding paper we demonstrate that Adriamycin interacts directly with myoglobin to alter the oxidation state of the heme-protein (21). It is feasible that Adriamycin cardiotoxicity reflects a decrement in oxygen storage capacity and/or oxidative damage attributable to this interaction of the drug with myoglobin. The purpose of the present investigation was to determine if Adriamycin stimulates myoglobin-dependent lipid peroxidation and whether oxygen free radicals are involved. The heart may be particularly sensitive to this type of damage because of the deficiency of antioxidant defense mechanisms in cardiac tissue (22).

MATERIALS AND METHODS

Reagents - The sources of the reagents and methods of preparing oxymyoglobin and purifying catalase are described in the preceding paper (21). Arachidonic acid (5,8,11,14-eicosatetraenoic acid; 90% pure from porcine liver) was purchased from Sigma Chemical Co. (St. Louis, MO). To minimize oxidation, arachidonic acid stock solutions were prepared upon receipt by dissolving 500 mg of arachidonic acid in 10 ml of absolute ethanol which had previously been bubbled for 30 min with argon at 0°C. The arachidonic acid in ethanol was then divided into equal aliquots in amber vials and each was purged with argon before being sealed and stored at -20°C in the dark until used (less than 1 month).

Experimental Methods - The experiments were designed to determine whether Adriamycin is able to stimulate myoglobin-dependent lipid peroxidation and also which oxidation state of myoglobin was most capable of stimulating lipid peroxidation. Each reaction mixture contained oxymyoglobin or metmyoglobin (2.5 μM, except where noted) and arachidonic acid (1 mg/ml) in 1 ml of 10 mM HEPES (pH 7.4). The incubation was at 37°C under air in a Dubnoff metabolic shaking incubator. Reactions were also incubated in the presence or absence of one or more of the

following: Adriamycin (50 μM except where noted), catalase (350 units/ml), SOD (8 μg protein/ml), various hydroxyl radical scavengers; mannitol (50 mM), sodium benzoate (25 mM), and 1,3-dimethylurea (10 mM), the iron chelators desferrioxamine (25 μM) and EDTA (25 μM), and ascorbate (1 mM). The reactions were started by adding arachidonic acid and stopped by the addition of 100 μl of 20 mM butylated hydroxytoluene (BHT) followed by vortex mixing. Zero-time reactions were stopped before adding arachidonic acid. As an indicator of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) were determined by the method of Buege and Aust (23).

Statistics - Each experiment was repeated at least three times and the data analyzed by analysis of variance. Differences between paired comparisons were considered to be statistically significant using Fisher's least significant difference method of analysis ($p<0.05$). In the case of Adriamycin-stimulated myoglobin-dependent lipid peroxidation, the effect of the inhibitors was determined to be statistically significant using the Students' paired t-test ($p<0.05$).

RESULTS

Time-Dependence of TBARS Formation - Both oxymyoglobin and metmyoglobin supported a 5 to 6-fold accumulation of lipid peroxidation products over a 3 h period (Fig. 1A and 1B, respectively). No TBARS were detected when incubations were conducted in the absence of

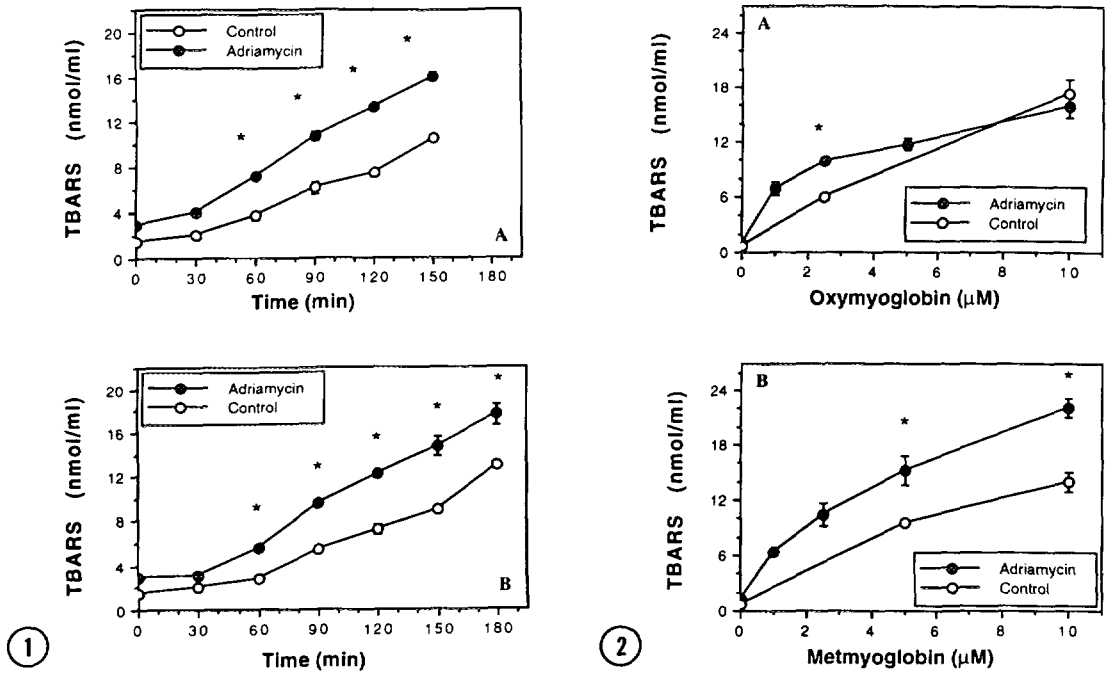
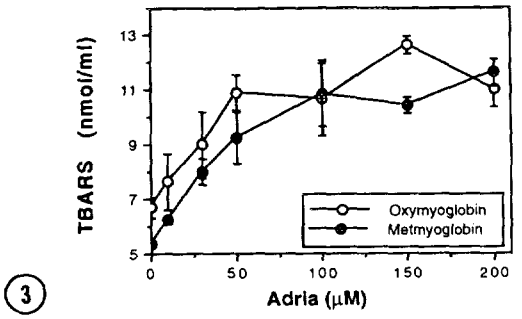
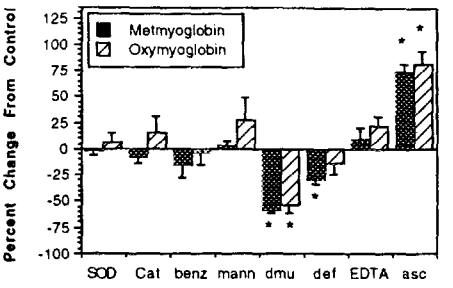


Figure 1. *Time-Dependence of Myoglobin-Dependent Lipid Peroxidation; Stimulation by Adriamycin.* Control samples contained 1 mg/ml arachidonic acid and either 2.5 μM oxymyoglobin (1A) or metmyoglobin (1B) in 10 mM HEPES (pH 7.4). Adriamycin (50 μM) was added as indicated. Lipid peroxidation was determined by measuring TBARS and the results expressed as the mean±SE for 3 repetitions performed on the same day. The asterisk (*) indicates a statistically significant difference from the control at each time point ($p<0.05$).

Figure 2. *Myoglobin-Dependent Lipid Peroxidation.* Controls contained 1 mg/ml arachidonic acid and various concentrations of either oxymyoglobin (2A) or metmyoglobin (2B) in 10 mM HEPES (pH 7.4). Adriamycin (50 μM) was added as indicated. Lipid peroxidation was estimated from TBARS formed during a 2 h incubation. Results are the mean±SE of 3 repetitions performed on the same day. The asterisk (*) indicates a statistically significant difference compared to the control at the respective heme protein concentration ($p<0.05$).



(3)



(4)

Figure 3. *Stimulation of Myoglobin-Dependent Lipid Peroxidation by Adriamycin.* Controls contained 1 mg/ml arachidonic acid and 2.5 μM metmyoglobin or oxymyoglobin in 10 mM HEPES (pH 7.4). Lipid peroxidation was determined by measuring TBARS formed during a 2 h incubation. Results are the mean±SE of 3 repetitions performed on the same day.

Figure 4. *Effect of Antioxidants on Adriamycin-Stimulated Myoglobin-Dependent Lipid Peroxidation.* Controls initially contained 1 mg/ml arachidonic acid, 50 μM Adriamycin, and 2.5 μM oxymyoglobin or metmyoglobin in 10 mM HEPES (pH 7.4). Additions of 350 units/ml catalase (Cat), 8 μg protein/ml SOD, 25 μM sodium benzoate (benz), 50 mM mannitol (mann), 25 μM desferrioxamine (def), 10 mM dimethylurea (dmu), 25 μM EDTA, and 1 mM ascorbate (asc) were as indicated in the figure. Lipid peroxidation was determined by measuring TBARS formed during a 2 h incubation. Results are presented as the percent change from the control sample (6.3-9.6 nmol TBARS/ml) which contained Adriamycin but no inhibitors ($9 \leq n \leq 24$). The asterisks (*) indicate a statistically significant difference from control ($p<0.05$).

either heme protein. Adding 50 μM Adriamycin significantly enhanced lipid peroxidation catalyzed by either oxy- or met-myoglobin. In the presence of either heme protein, and after two hours of incubation, the drug caused an approximate doubling in the concentration of lipid peroxidation products compared to controls incubated in the absence of Adriamycin.

Myoglobin-Dependence of TBARS Formation - Both oxymyoglobin and metmyoglobin catalyzed the peroxidation of arachidonic acid in the absence of Adriamycin (Fig. 2). In either case, the extent of lipid peroxidation was dependent on the concentration of the heme protein. Depending on the protein, Adriamycin elicited a 50-70% stimulation of lipid peroxidation. The stimulation of lipid peroxidation by Adriamycin was significant at 2.5 μM, but not 10 μM oxymyoglobin whereas with metmyoglobin, statistical significance was achieved at concentrations above 5 μM. There was no apparent difference in the ability of oxymyoglobin and metmyoglobin to catalyze lipid peroxidation.

Adriamycin-Dependence of TBARS Formation - Adriamycin concentrations of 30 μM and higher caused a significant stimulation of TBARS production in the presence of either oxymyoglobin or metmyoglobin (Fig. 3). With either heme protein, TBARS production reached a maximum of approximately 11 nmol/ml at 50 μM Adriamycin. However, no peroxidation was detected when arachidonic acid was incubated with Adriamycin alone, illustrating the strict dependence of the reaction on myoglobin (data not shown).

Affect of Modifying Agents on Lipid Peroxidation - The affect of various modifying agents on Adriamycin-stimulated, myoglobin-dependent peroxidation of arachidonic acid is presented graphically in figure 4. In the presence of either oxy- or met-myoglobin, lipid peroxidation was significantly inhibited by dimethylurea and stimulated by ascorbate. Desferrioxamine inhibited

lipid peroxidation in both preparations although this effect was only significant in the reaction containing metmyoglobin. The addition of catalase, SOD, sodium benzoate, mannitol, or EDTA had no significant effect on Adriamycin-induced lipid peroxidation catalyzed by either oxy- or met-myoglobin.

DISCUSSION

Reactive oxygen intermediates alter the oxidation state, and thus the oxygen-carrying capacity of the heme-containing proteins myoglobin and hemoglobin (20,21). Although the interaction of reactive oxygen species with heme-containing proteins is likely to elicit many reactions, one of the most well-studied is lipid peroxidation (16,24). The demonstrated stimulation of myoglobin-dependent lipid peroxidation by Adriamycin provides new insight into the mechanism by which the drug may be selectively cardiotoxic.

The lack of effect of the oxygen radical scavengers SOD, catalase, benzoate, and mannitol suggest that myoglobin-catalyzed lipid peroxidation in the presence of Adriamycin does not involve liberation of free radical intermediates of oxygen. This finding is supported by Gianni *et al.* (7) who observed that catalase, SOD and mannitol have no affect on microsomal lipid peroxidation catalyzed by Adriamycin-iron chelates. The failure of mannitol and benzoate to inhibit Adriamycin-stimulated lipid peroxidation is consistent with a number of investigations demonstrating that lipid peroxidation catalyzed by activated-iron, both free and as myoglobin or hemoglobin, does not involve the hydroxyl radical (17,24,25). However, this interpretation is complicated by the profound inhibition of lipid peroxidation by dimethylurea. It has been suggested that the ability of dimethylurea to inhibit reactions involving hydroxyl radicals despite the ineffectiveness of other hydroxyl radical scavengers is indicative of the extreme instability of these free radicals, which react rapidly and nonspecifically with cellular nucleophiles in the immediate vicinity of the site of generation (26,27). Selective inhibition by dimethylurea may reflect its distinct ability to gain access to the site of hydroxyl radical production, a consequence of its greater lipid solubility and/or smaller molecular radius compared to mannitol and sodium benzoate (26,27).

The limited inhibitory effects of desferrioxamine and EDTA indicate that free iron plays a minor role in these reactions. While heme-catalyzed lipid peroxidation leads to the eventual decomposition of the protein and ultimately to iron release (25), we failed to detect iron release from myoglobin in any of the reactions examined (21). Therefore, while the slight inhibition of lipid peroxidation by desferrioxamine may be due to chelation of trace metal ions in the reaction mixture, a more likely explanation is derived from the work of Rice-Evans *et al.*, (18) and Kanner and Harel (28). Using similar model systems, both groups demonstrated that desferrioxamine inhibits reactions involving reactive oxygen species by acting as a reducing agent, an effect unrelated to its iron-chelating ability. Desferrioxamine does not extract iron from myoglobin (29).

Ascorbate profoundly stimulated lipid peroxidation catalyzed by either oxymyoglobin or metmyoglobin in the presence of Adriamycin. While ascorbate can function as an antioxidant, it is also reported to stimulate iron-dependent lipid peroxidation, purportedly by reducing ferric iron (28,30,31). Such a mechanism has been implicated for the ascorbate-induced stimulation of metmyoglobin-dependent lipid peroxidation (32).

In light of our results with the various modifying agents, no obvious involvement of oxygen free radicals in the stimulation of myoglobin-dependent lipid peroxidation can be implied and the mechanism appears to be complex. Myoglobin may stimulate lipid peroxidation by catalyzing the breakdown of pre-formed lipid peroxides, present in all cell membranes, to reactive alkoxy and peroxy lipid radicals. This hypothesis is consistent with our finding that oxy- and met-myoglobin were equivalent in their catalysis of lipid peroxidation. Small differences in the ability of oxy- or metmyoglobin to initiate lipid peroxidation may be of little significance since the propagation reactions are responsible for over 90% of the lipid peroxidation observed (33).

The role of Adriamycin in this mechanism may resemble that proposed for Hg^{+2} and Ag^{+2} in hemoglobin-dependent lipid peroxidation. Both are believed to act by facilitating the binding of oxy- and methemoglobin to liposomes, an event reported to be essential for heme catalysis of lipid peroxidation (34,35). Alternatively, Gutteridge (6) observed that stimulation of iron-dependent lipid peroxidation by Adriamycin does not involve a change in the oxidation state of the drug and suggested that Adriamycin acts by facilitating electron transfer from iron to the lipid. While we observed no evidence for the complexation of Adriamycin and myoglobin, it is possible that in the presence of lipid, conformational changes occur in the structure of myoglobin which facilitate the transfer of electrons between the heme-iron, drug, and lipid.

In summary, Adriamycin stimulates myoglobin-dependent lipid peroxidation by a mechanism which may involve the hydroxyl radical. The heart may be uniquely sensitive to this type of oxidative damage for two reasons. First, heart tissue lacks the antioxidant enzymes present in metabolic organs such as the liver and kidney (22). Second, cardiac tissue represents an abundant source of myoglobin, which is required to sustain the contractile function of this organ. While we do not discount oxidative damage to other myoglobin-containing tissues, damage to this vital organ is likely to be of greater consequence to the health of the individual. Accordingly, interference with myoglobin-dependent oxygen transport and stimulation of lipid peroxidation by Adriamycin, may combine to be significant determinants of the cardiotoxicity of this drug.

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