Free Rad. Res. Comms., Vols. 12-13, pp. 107-114
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FERRITIN, LIPID PEROXIDATION AND REDOX-CYCLING XENOBIOTICS

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A number of xenobiotics are toxic because they redox cycle and generate free radicals. Interaction with iron, either to produce reactive species such as the hydroxyl radical, or to promote lipid peroxidation, is an important factor in this toxicity. A potential biological source of iron is ferritin. The cytotoxic pyrimidines, dialuric acid, divicine and isouramil, readily release iron from ferritin and promote ferritin-dependent lipid peroxidation. Superoxide dismutase and GSH, which maintain the pyrimidines in their reduced form, enhance both iron release and lipid peroxidation. Microsomes plus NADPH can reduce a number of iron complexes, although not ferritin. Reduction of Adriamycin, paraquat or various quinones to their radicals by the microsomes enhances reduction of the iron complexes, and in some cases, enables iron release from ferritin. Adriamycin stimulates iron-dependent lipid peroxidation of the microsomes. Ferritin can provide the iron, and peroxidation is most pronounced at low pO_2. Complexing agents that supress intracellular iron reduction and lipid peroxidation may protect against the toxicity of Adriamycin.

KEY WORDS: Ferritin, lipid peroxidation, iron, Adriamycin, microsomes, dialuric acid.

ABBREVIATION: SOD; superoxide dismutase

INTRODUCTION

Lipid peroxidation can be described in terms of three processes: initiation involving hydrogen abstraction from a polyunsaturated fatty acid; propagation of the radical chain;

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \qquad LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$

and termination involving biradical reactions.[1-3] However, other reactions involving breakdown of unstable intermediates and products, e.g. LOOH, also occur and may play a greater role in more complex biological systems.

Many systems that cause peroxidation of isolated liposomes or microsomes have a requirement for a transition metal, usually iron. Until recently, iron was generally considered to participate via the Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

followed by hydrogen abstraction by the $OH^{\cdot}$ produced. However, in many studies, catalase enhances rather than inhibits peroxidation, with the only other requirements being O_2 and a reducing system that may or may not be $O_2^{\cdot-}$.[1-3] Suggestions for the reactive species include ferryl[4] and $Fe^{2+}/Fe^{3+}/O_2$ complexes.[1,5] However, whatever its

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identity, it appears that in these systems, peroxidation does not depend on hydrogen abstraction from an unsaturated lipid for initiation, but on the breakdown of pre-existing lipid hydroperoxides catalysed by the iron complex.[2] Extremely low amounts of hydroperoxide are all that is needed for this mechanism to predominate. This breakdown produces lipid radicals, that then participate in chain propagation reactions.

For this mechanism to occur physiologically, it would require a source of redox-active iron and a reducing system. Although physiological concentrations of low Mr iron complexes are very low, iron is stored intracellularly as ferritin. Recent studies have shown that a variety of radical-generating systems can release iron from ferritin, suggesting that it may be a source of iron for promoting biological damage such as lipid peroxidation.[6] However, the released iron must be in a form capable of undergoing the redox reactions involved in the peroxidation sequence.

This presentation considers mechanisms of iron reduction and ferritin-iron release by different radical-generating systems, and consequent lipid peroxidation. Two types of redox-cycling xenobiotics are discussed. The first are readily autoxidisable compounds e.g. dialuric acid (2,4,5,6-tetrahydroxypyrimidine) and the related 2-amino- and 2,6-diamino- compounds, isouramil and divicine, and 6-hydroxydopamine. These compounds generate radicals and H_2O_2 on autoxidation. Dialuric acid is the reduced form of alloxan, a potent pancreatic islet cell toxin. Isouramil and divicine are the compounds, present in fava beans as their glycosides, that cause red cell destruction in favism. The second group of compounds includes quinones and anthracycline anti-cancer drugs like Adriamycin. These compounds undergo metabolic reduction to form radicals, which can subsequently redox cycle by reaction with O_2.

FERRITIN-IRON RELEASE AND LIPID PEROXIDATION BY AUTOXIDISABLE COMPOUNDS

Iron is stored within ferritin as a ferric oxyhydroxide polymer that requires reduction for release. How this occurs *in vivo* is not known, since the reaction of ferritin with known physiological reducing agents e.g. ascorbate, is very slow.[6,7] However, a number of strongly reducing xenobiotics release ferritin-iron rapidly. These include dialuric acid, isouramil and divicine (Table I), which are at least 10 time more reactive

TABLE I
Iron release from ferritin and lipid peroxidation promoted by dialuric acid, isouramil and divicine

	Ferritin-iron release (μM/30 min)			Lipid peroxidation (A_{532})	
	N_2	air	air + GSH/SOD	air	air + GSH/SOD
dialuric acid	30	0.98	19.9	0.30	0.71
isouramil	12	0.50	8.6	0.22	0.92
divicine	17	0.73	6.3	0.32	0.73

Ferritin-iron release was measured at 37°C in phosphate-buffered saline pH 7.3 containing ferrozine (200 μM), ferritin (200 μg/ml), each pyrimidine (100 μM) and where indicated, SOD (10 μg/ml) and GSH (600 μM). The increase in A_{562} due to formation of Fe^{2+} (ferrozine) was monitored. Lipid peroxidation was monitored under similar conditions, with sheep brain phospholipid liposomes (1 mg/ml), ferritin (50 μg/ml) and pyrimidine (100 μM). Thiobarbituric acid-reactive substances were measured at 532 nm after 30 min incubation. Results are taken from ref[8].

than ascorbate.[8] 6-Hydroxydopamine is about 10 times more effective than the pyrimidines.[9] The iron is released as Fe^{2+}, and can be detected by forming a coloured complex with bipyridyls.

The pyrimidines autoxidise rapidly in air, predominantly by a O_2^--dependent chain reaction in which the xenobiotic radical is an intermediate.[10] Release of ferritin-iron is very much less in air than in N_2, when autoxidation is prevented (Table I). This suggests that it is the reduced form of each compound, rather than radicals generated from it, that releases iron directly. Consistent with this, adding GSH and superoxide dismutase (SOD), which scavenge the radical intermediates and prevent autoxidation,[11] enhances the amount of released iron (Table I). Alloxan is reduced by GSH and behaves identically to dialuric acid.

Addition of the pyrimidines to liposomes induces lipid peroxidation. Peroxidation is detectable in the presence of traces of adventitious iron, but is enhanced in the presence of ferritin.[8,9] The reaction is not inhibited by catalase or SOD, i.e. it probably proceeds via the mechanism involving lipid hydroperoxide breakdown described above, in which the pyrimidines themselves provide the reducing equivalents. Peroxidation is inhibited by desferrioxamine, which indicates that it involves released iron. Lipid peroxidation is increased by GSH alone, and even more by GSH in combination with SOD (Table I). Thus the maintenance of the pyrimidines in a reduced form and the increased release of ferritin-iron are associated with enhanced lipid peroxidation.

These cytotoxic xenobiotics, therefore, can release iron from ferritin directly, and this could be a factor in their toxicity. GSH and SOD suppress autoxidation and the generation of O_2^- and H_2O_2. Although this has been proposed as a protective mechanism against toxicity,[11,12] it is clear that the combination does not protect against, and in fact enhances, ferritin-iron release and consequent lipid peroxidation.

RADICAL-MEDIATED IRON RELEASE FROM FERRITIN

A wide variety of compounds that undergo metabolic reduction release iron from ferritin by a radical-mediated process (see examples in Table II).[6,13-15] O_2^- has this ability.[6,13] This reaction may contribute to O_2^- toxicity, although under most conditions the iron release corresponds to only a few percent of the O_2^- generated. On the

TABLE II
Rates of iron release from ferritin by O_2^- and xenobiotic reducing radicals

Parent Compound	Iron Release (μM/min)
O_2 (O_2^-)	0.06
Paraquat	1.98
Diquat	1.14
Adriamycin	1.70
1-Hydroxyanthraquinone	1.20
Menadione	0
Napthoquinone	0
Benzoquinone	0

Radicals were generated at a uniform rate of 6 μM/min, by incubating the parent compounds with hypoxanthine and xanthine oxidase at 25°C in phosphate buffer pH 7.4. O_2^- was generated in air, the other radicals were generated in N_2. Iron release was monitored as an increase in A_{562} in the presence of 200 μM ferrozine. Results are taken from ref[15].

other hand, some reducing radicals, e.g. paraquat radicals, can give almost stoichiometric iron release.

Not all reducing radicals that can be generated enzymatically release iron from ferritin. The semiquinones of benzoquinone and menadione, for example, are inactive (Table II). Reactivity appears not to depend on charge, or on size since radicals too large to enter the pores in the protein shell are good releasers. Reduction potential appears to be the major determinant,[15] with a cutoff observed at about -0.24 V. This is close to the reduction potential of ferritin at pH 7.4.[16]

The rates shown in Table II were measured anaerobically. They fall considerably with the introduction of air, reflecting the reaction of the organic radicals with O_2 to give O_2^- and its less efficient reaction with ferritin. This implies that ferritin-iron release mediated by quinone-type compounds will be greatest under conditions that favour the semiquinone over O_2^-. The most efficient release should occur, therefore, in hypoxic cells or cells made hypoxic through redox cycling.

MICROSOMAL IRON REDUCTION

Microsomes undergo lipid peroxidation in the presence of NADPH and iron or iron(ADP) complexes.[17,18] Peroxidation is enhanced by some xenobiotics e.g. Adria-

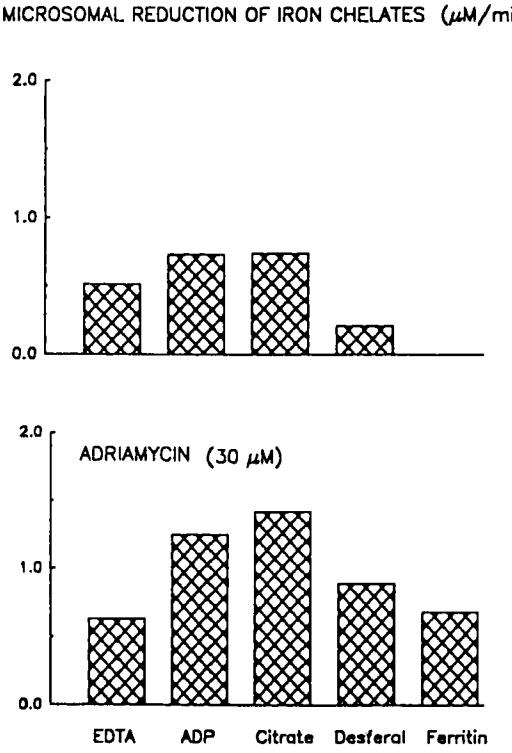

FIGURE 1 Rates of reduction of Fe^{3+} chelates by rat liver microsomes and NADPH in the absence and presence of Adriamycin. The chelates (10 μM Fe^{3+}, 0.2 or 1mM chelator) and ferritin (50 μg/ml) were incubated at 37° C, pH 7.4 under N_2 with microsomes (0.43 mg/ml) and NADPH (100 μM). Iron reduction was measured as an increase in A_{562} in the presence of ferrozine (100 μM). Results are taken from ref[20].

mycin.[19,20] Although the requirement for NADPH indicates a reductive step involving cytochrome P450 reductase, the mechanism has not until recently become clear, since the Fe^{3+} complexes that participate in lipid peroxidation are not reduced by the purified reductase.[18,21] Fe^{3+}(EDTA) is reduced but does not promote peroxidation.

However, a more extensive range of complexes, including Fe^{3+}(ADP), is reduced by intact microsomes (Figure 1, upper panel).[22] It is noteworthy that Fe^{3+}(desferal), frequently considered to be redox-inert, is reduced by the microsomal system. Ferritin-iron, however, is not. Reduction rates are much higher in N_2 than in air.[22] They decrease progressively with increasing concentration of O_2, indicating that reduction is not mediated by O_2^-. CO also inhibits, although this can be overcome by increasing the concentration of iron. These findings suggest that the complexes are reduced directly by cytochrome P450, probably by the ferrous form, in competition with its reacting with O_2 or CO.

In the presence of compounds such as Adriamycin (Figure 1), paraquat, menadione or anthraquinone-2-sulphonate, NADPH oxidation and reduction of most of the iron chelates are enhanced, and all but menadione reduce ferritin-iron as well.[22] These reactions are inhibited by O_2, but reduction mediated by xenobiotics does not appear to require cytochrome P450. It most likely involves reduction of the Fe^{3+} complexes

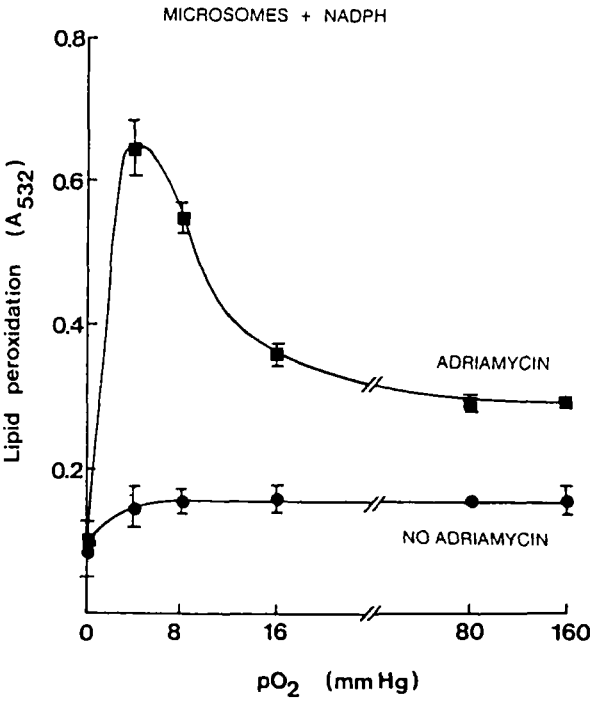

FIGURE 2 Peroxidation of rat liver microsomes as a function of pO_2 in the presence and absence of Adriamycin (30 μM). Microsomes (0.43 mg/ml) were incubated in phosphate buffer pH 7.4 at 22°C for 30 min with NADPH (100 μM) and $FeCl_3$ (1 μM). Peroxidation was measured as thiobarbituric acid reactivity at 532 nm. Reproduced with permission.[22]

by the xenobiotic radicals produced by P450 reductase. O_2 inhibits because it reacts with the radicals to give O_2^-, which is a much less efficient iron reductant.

Thus, we have been able to identify microsomal mechanisms for reducing iron complexes under conditions where they promote lipid peroxidation. However, although iron reduction may be necessary, it is not sufficient for peroxidation to occur. Some complexes, e.g. Fe(EDTA) and Fe(desferrioxamine), are reduced without causing peroxidation, and of the xenobiotics we have studied, only Adriamycin gives marked enhancement of the process.

ADRIAMYCIN-DEPENDENT LIPID PEROXIDATION

Adriamycin-dependent lipid peroxidation occurs in the presence of catalytic concentrations of iron and a reducing system.[19,20,23] Microsomal peroxidation is NADPH-dependent. There has been a lack of consensus regarding the involvement of O_2^- and $OH^{\cdot}$, with some authors, but not others, reporting inhibition by catalase and SOD. A complication is the presence of both these enzymes (and ferritin) in most microsomal preparations.[13] When these are removed, it is apparant that H_2O_2 inhibits rather than stimulates peroxidation, O_2^- is not essential, and the initial reaction is probably the breakdown of lipid hydroperoxides as described above.[22]

A major feature of Adriamycin-dependent peroxidation of microsomes is that it occurs 3–4 times more efficiently at a pO_2 of 5–10 mm Hg than it does in air (Figure 2). This is a consequence of the efficiency of iron reduction decreasing with increasing pO_2, as more of the Adriamycin semiquinone is converted to O_2^-. Some O_2 is required, however, to propagate the peroxidative chain and maximum peroxidation is seen when the two opposing effects of O_2 on initiation and propagation balance.

In keeping with our iron reduction measurements, ferritin is able to catalyse microsomal lipid peroxidation in the presence but not absence of Adriamycin.[22]

The O_2 concentrations at which we observed maximal lipid peroxidation occur in many tissues including active muscle cells. Oxidative damage, possibly involving lipid peroxidation, is thought to be involved in the cardiotoxicity of Adriamycin. The cellular pO_2 may be a factor in this toxicity. Our results show that the necessary iron not only for lipid peroxidation, but also for other iron-dependent damaging processes, can be provided by ferritin. Its release also will be favoured at low pO_2.

Minimising the cardiotoxicity of Adriamycin is obviously advantageous for its use as an anti-cancer agent. A drug that has shown promise clinically in this respect, when administered prior to Adriamycin therapy, is ICRF-187.[24,25] This compound (*d*-1,2-bis(3,5-dioxopiperazin-1-yl) propane) hydrolyses intracellularly to give ICRF-198 (*dl*-NN'-dicarboxamidomethyl-NN'-dicarboxymethyl-1,2-diaminopropane), a chelating agent similar to EDTA.[26] The effectiveness of ICRF-187 may therefore be releated to iron chelation. If so, it should not only have a high affinity for iron, but also form a complex that does not readily undergo reduction or promote Adriamycin-dependent lipid peroxidation. We have found this to be the case. ICF-198 complexes iron in competition with a large excess of ADP, and the Fe^{3+} complex is reduced by microsomes and Adriamycin at only 20% the rate of Fe^{3+}(ADP) reduction. It strongly inhibits lipid peroxidation, and suppresses the inactivation of microsomal CaATPase, catalysed by iron or iron(ADP). It does not suppress iron release from ferritin, but does prevent the released iron from participating in these subsequent oxidations.

We conclude from these results that ICRF-198 could trap intracellular iron in a poorly reactive form, and thus protect against oxidative damage by Adriamycin. The observed protection by ICRF-187, therefore, may be some of the strongest evidence available that oxidants and iron are involved in the cardiotoxicity of Adriamycin.

Acknowledgements

This work was supported by grants from the Medical Research Council of New Zealand and the Cancer Society of New Zealand.

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Accepted by Prof. G. Czapski