Biochemical Pharmacology, Vol. 37, No. 15, pp. 2893–2897, 1988.
Printed in Great Britain.

0006–2952/88 $3.00 + 0.00

ADRIAMYCIN-DEPENDENT PEROXIDATION OF RAT LIVER AND HEART MICROSOMES CATALYSED BY IRON CHELATES AND FERRITIN

MAXIMUM PEROXIDATION AT LOW OXYGEN PARTIAL PRESSURES

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(*Received* 21 *October* 1987; *accepted* 25 *January* 1988)

Abstract—NADPH- and iron-dependent lipid peroxidation of rat heart and liver microsomes was measured in the presence and absence of adriamycin. Lipid peroxidation was enhanced by adriamycin when incubated in air and was increased as the pO_2 was lowered, to a maximum of 3–4 times the aerobic level at a pO_2 of approx. 4 mm Hg. Fe-ADP, Fe-ATP and ferritin were able to catalyse adriamycin-dependent peroxidation of microsomes under low pO_2. Superoxide dismutase and catalase had minimal effect. These results indicate that adriamycin-dependent lipid peroxidation is favoured by the low O_2 concentrations that exist in active muscle cells and suggest that ferritin could provide the iron catalyst for the reaction.

The effectiveness of adriamycin as an anticancer drug is limited by its dose-dependent cardiotoxicity. Although the mechanism of cardiotoxicity is not fully understood, there is mounting evidence that reduction of adriamycin to the semiquinone and subsequent redox cycling with O_2 are involved [1–4]. The two main intracellular sites of adriamycin reduction are mitochondrial NADH reductase and microsomal NADPH-cytochrome P-450 reductase [3–6].

Lipid peroxidation has been detected following adriamycin administration in some *in vivo* studies [7, 8], although not in others [9, 10]. Adriamycin has been shown to enhance NADPH-dependent lipid peroxidation in isolated liver and heart microsomes from various species. However, these *in vitro* studies were carried out in O_2 [2, 11] or in air [12, 13]. O_2 concentrations within functioning heart cells are much lower. Their pO_2 is typically 5–10 mm Hg (0.7–1.4% O_2) [14], and O_2 consumption by redox cycling of adriamycin would decrease these levels even further.

Adriamycin semiquinone reacts with H_2O_2 and certain iron chelates to produce a highly reactive oxidant, possibly the hydroxyl radical or a ferryl species [15–18]. This Fenton reaction is inhibited by O_2 because it competes with the ferric catalyst for the adriamycin semiquinone, forming superoxide which is much less efficient at recycling the iron catalyst [17–19]. Furthermore, peroxidation of liposomes prepared from ox brain phospholipid, induced by xanthine, xanthine oxidase, adriamycin and catalytic iron, is optimal in solutions maintained at a pO_2 of only 5–10 mm Hg [18]. These findings imply that microsomal lipid peroxidation might be greater at the more physiological low O_2 concentrations. Therefore, we have measured the O_2 dependence of adriamycin-dependent lipid peroxidation in rat liver and heart microsomes. We have also determined the efficiencies of different iron chelates, including ferritin, of catalysing the reaction and whether the mechanism of peroxidation involves superoxide or hydrogen peroxide.

MATERIALS AND METHODS

Liver [20] and heart [21] microsomes were prepared from 3-month-old Sprague–Dawley rats, and resuspended in 10 mM phosphate buffer, pH 7.4. For some experiments, liver microsomes were further purified by passing down a 2.5 × 30 cm column packed with Sepharose CL-2B and eluting with the same buffer as was used for preparing the microsomes. This method has been shown to separate loosely associated proteins such as superoxide dismutase, catalase and ferritin from the microsomes [22]. Protein content of the preparations was determined according to Lowry *et al.* [23]. All procedures were carried out using acid-washed glassware and buffers treated with chelex resin (BioRad Laboratories, California) to minimise iron contamination. Biochemicals were from Sigma (St Louis, MO). The ferritin, used as supplied, contained 1.04 nmoles iron/μg. This was determined by releasing the iron by the method of Hoy *et al.* [24], adding ferrozine and measuring A_{562} [25].

Microsomes (0.43 mg liver or 0.50 mg heart) were incubated in 1 ml of 10 mM phosphate buffer with 100 μM NADPH, 30 μM adriamycin (Farmitalia Carlo Erba, Italy) and either $FeCl_3$ plus chelator or ferritin. Chelators were added to the iron before addition of other reactants. Reactions were performed in 10 ml glass tubes with air-tight rubber stoppers. Solutions were bubbled with O_2-free N_2 for 2 min and the pO_2 was adjusted by adding the

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required volume of air to each tube with a gas-tight syringe, after removal of an equivalent volume of N_2. For example, to give a pO_2 of 4 mm Hg, 0.25 ml of air was added. Incubations were for 30 min at 22° on a roller so that solutions were thoroughly equilibrated with the gaseous phase. Lipid peroxidation was measured as thiobarbituric acid (TBA) reactivity [18].

Cytochrome P-450 reductase activity was measured by incubating 0.5 and 0.43 mg of heart and liver microsomes respectively with 100 μM NADPH and 25 μM cytochrome *c* and monitoring ΔA_{550}.

RESULTS

Rat liver microsomes

Adriamycin has been shown to enhance NADPH-dependent microsomal lipid peroxidation in air and in O_2 [2, 11–13]. As shown in Fig. 1, microsomes equilibrated with a pO_2 of 4 mm Hg (0.5% O_2) underwent lipid peroxidation, measured as TBA-reactive products, which was increased in the presence of adriamycin. Lipid peroxidation increased with increasing iron concentration in the micromolar range (Fig. 1). The yield with no iron added probably reflects catalysis by adventitious transition metals present in the reagents. A_{532} values were read against blanks incubated without NADPH. With adriamycin and 1 μM Fe, blank values were approx. 10% of the total product yield. Since this would include breakdown of pre-existing lipid peroxides, such breakdown did not contribute significantly to total peroxidation.

The O_2 dependence of peroxidation, carried out in the presence of 30 μM adriamycin and 1 μM Fe, showed a sharp peak of TBA-reactive products at a

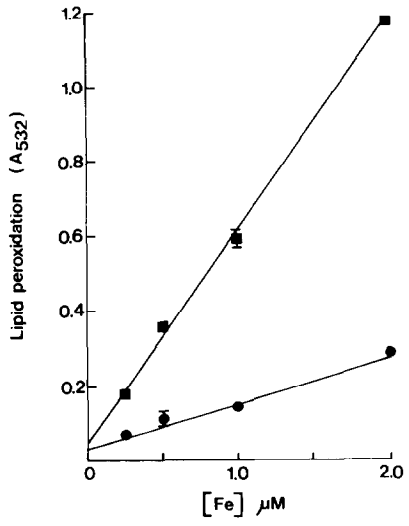

Fig. 1. Dependence of rat liver microsomal lipid peroxidation on iron concentration. Microsomes were incubated with (■) and without (●) 30 μM adriamycin and NADPH at a pO_2 of 4 mm Hg as described in the Materials and Methods section. Final concentrations of $FeCl_3$ are shown. Each point is the mean ± SD for two sets of duplicates and no error bars are shown where the SD is within the symbol height.

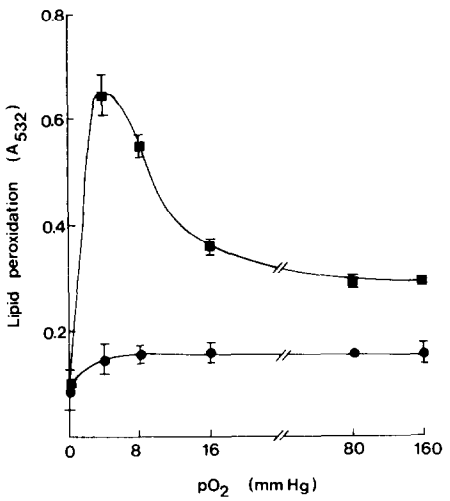

Fig. 2. Dependence of liver microsomal lipid peroxidation on pO_2. Microsomes were incubated with (■) and without (●) 30 μM adriamycin, NADPH and 1 μM $FeCl_3$ as described in the Materials and Methods section. Each point is the mean ± SD for two sets of duplicates and no error bars are shown where the SD is within the symbol height.

pO_2 of 4 mm Hg (Fig. 2). There was no such peak in the absence of adriamycin.

Lipid peroxidation was substantially enhanced when the iron was complexed with ADP or ATP, but was strongly inhibited by citrate, EDTA and desferrioxamine (Table 1). Maximum inhibition was seen with 50 μM EDTA or desferrioxamine, but in excess of 1 mM was required for maximal effect with the other chelators. Adriamycin-dependent lipid peroxidation catalysed by Fe(ADP) or Fe(ATP) was also 2–3 times greater at low pO_2 than in air (Table 1).

Microsomes, in the presence of NADPH and adriamycin, can release iron from ferritin [26; Vile and Winterbourn, in preparation]. As shown in Table 2, we found that addition of ferritin to NADPH, adriamycin and microsomes resulted in lipid peroxidation which was substantially greater at a pO_2 of 4 mm Hg than in air. Peroxidation was not enhanced in the presence of ADP but it was inhibited by EDTA. No lipid peroxidation was observed in the absence of adriamycin, which is in accordance with the lack of iron release from ferritin under these conditions [26; Vile and Winterbourn, in preparation]. Adriamycin-dependent lipid peroxidation increased with increasing ferritin concentration up to a maximum with 25 μg/ml (Fig. 3). The TBA-reactive product yield with 15 μg/ml ferritin (15.6 μM ferritin iron) was equivalent to that with 1 μM free iron.

Adriamycin-dependent lipid peroxidation, measured under the same conditions as for Fig. 2, did not require superoxide or hydrogen peroxide. Superoxide dismutase (60 μg/ml) inhibited TBA-reactive product formation by 5 ± 1% in air and 10 ± 2% at a pO_2 of 4 mm Hg. The corresponding values for inhibition by catalase (20 μg/ml) were 2 ± 1% and 4 ± 1%. Values are means and ranges from 3 or 4 estimations.

Table 1. Effect of iron and iron-chelates on rat liver microsomal lipid peroxidation

Chelator	A_{532}	
	pO_2 = 160 mm Hg	pO_2 = 4 mm Hg
None	0.15 ± 0.01	0.62 ± 0.05
ATP (1 mM)	0.31 ± 0.02	0.89 ± 0.04
ADP (1 mM)	0.38 ± 0.04	0.81 ± 0.03
Citrate (1 mM)	0.08 ± 0.01	0.12 ± 0.03
EDTA (100 μM)	0.06 ± 0.01	0.10 ± 0.02
Desferrioxamine (100 μM)	0.01 ± 0.01	0.02 ± 0.01

Solutions all contained 1 μM $FeCl_3$, NADPH and adriamycin as described in Materials and Methods section. Figures are means ± SD for 4 estimations.

Table 2. Effect of ferritin on rat liver microsomal lipid peroxidation

Addition	A_{532}	
	pO_2 = 160 mm Hg	pO_2 = 4 mm Hg
None	0.03 ± 0.005	0.02 ± 0.005
Adriamycin	0.23 ± 0.02	0.85 ± 0.04
Adriamycin + ATP (1 mM)	0.24 ± 0.01	0.83 ± 0.04
Adriamycin + EDTA (100 μM)	n.d.	0.09 ± 0.01

Solutions all contained ferritin (50 μg), NADPH and microsomes as described in Materials and Methods section. Figures are means ± SD for 4 estimations, n.d. not determined.

Purification of the microsomes by gel filtration and reconstitution to an equivalent volume decreased their content of superoxide dismutase and catalase 10- and 6-fold respectively, but did not alter the amount of adriamycin-dependent lipid peroxidation, measured at low pO_2 of 4 mm Hg. The effect of adding superoxide dismutase or catalase was essentially the same as with the unpurified microsomes (11 ± 1% and 5 ± 1% inhibition respectively).

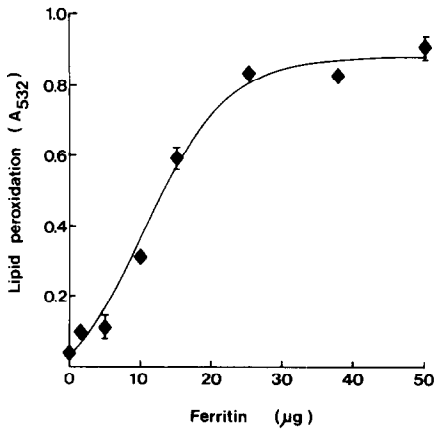

Fig. 3. Effect of ferritin on liver microsomal lipid peroxidation. Microsomes were incubated with adriamycin, NADPH and increasing concentrations of ferritin as described in the Materials and Methods section. No error bars are shown where the SD is within the symbol height.

Rat heart microsomes

Heart microsomes underwent a small amount of NADPH-dependent lipid peroxidation in the presence of 1 μM iron that was increased with 30 μM adriamycin present (Table 3). Adriamycin-enhanced peroxidation was greater at a pO_2 of 4 mm Hg than in air and was affected by iron chelators in the same way as for liver microsomes (Table 3). TBA-reactive product yields (with or without adriamycin) were only about one-tenth of those with liver microsomes (cf. Tables 1 and 3). This can be explained, at least in part, by the low P-450 reductase activity of the rat heart microsomes (0.93 nmol cytochrome *c* reduced/min/mg protein) in comparison to the activity of rat liver microsomes (7.5 nmol/min/mg). Superoxide dismutase scarcely affected TBA-reactive product formation (2 ± 1% inhibition in air and 8 ± 4% at a pO_2 of 4 mm Hg for 3 experiments). Catalase gave <2% inhibition at both O_2 concentrations.

DISCUSSION

Enhancement of microsomal lipid peroxidation by adriamycin is well established [2, 11–13]. The present study agrees with previous findings that this is iron-dependent [27, 28] and shows that there is much greater enhancement at low O_2 concentrations than in air, with optimal peroxidation occurring at approx. 4 mm Hg (0.5% O_2). This pO_2 optimum was observed with rat liver and heart microsomes in the presence of $FeCl_3$, Fe(ADP), Fe(ATP) or ferritin. It is not an artefact of the TBA assay, since adriamycin-independent peroxidation gave no such optimum,

Table 3. Effect of adriamycin, iron and iron-chelates on rat heart microsomal lipid peroxidation

Addition	A_{532}	
	pO_2 = 160 mm Hg	pO_2 = 4 mm Hg
None	0.012 ± 0.001	0.018 ± 0.001
Adriamycin	0.019 ± 0.002	0.078 ± 0.01
Adriamycin + ATP (1 mM)	n.d.	0.108 ± 0.005
Adriamycin + EDTA (100 μM)	0.005 ± 0.001	0.008 ± 0.001
Adriamycin + citrate (1 μM)	n.d.	0.010 ± 0.002

Solutions all contained 1 μM $FeCl_3$, NADPH and heart microsomes as described in the Materials and Methods section. Figures are means ± SD for 4 estimations, n.d. not determined.

and peroxidation of liposomes by Fe/ascorbate progressively decreases with decreasing pO_2 [18].

A low pO_2 optimum has also been observed for adriamycin-dependent peroxidation of liposomes by a hypoxanthine/xanthine oxidase system [18], and could arise through opposing effects on initiation and propagation of the peroxidation chain. Adriamycin-dependent peroxidation of microsomal lipid requires cytochrome P-450 reductase activity and iron, implying that iron reduction is required for initiation. We have shown that microsomes and NADPH can reduce Fe^{3+} and chelates such as Fe^{3+}(ADP) and Fe^{3+}(ATP) directly (Vile and Winterbourn, in preparation). Reduction is greatly enhanced by adriamycin (Adr), presumably because it proceeds via the semiquinone ($Adr^{\cdot}$):

$$NADPH + 2Adr \xrightarrow[\text{reductase}]{P_{450}} 2\,Adr^{\cdot} + NADP^+ + H^+ \quad (1)$$

$$Adr^{\cdot} + Fe^{3+}\,(\text{chelate}) \longrightarrow Adr + Fe^{2+}\,(\text{chelate}) \quad (2)$$

O_2 inhibits, presumably forming superoxide

$$Adr^{\cdot} + O_2 \rightleftharpoons Adr + O_2^{\cdot-} \quad (3)$$

which is a much poorer iron reductant [19]. Thus inhibition of microsomal iron reduction would explain why O_2 inhibits initiation of lipid peroxidation, and this, plus the O_2 dependence of propagation, would explain the low O_2 optimum.

Lack of inhibition by superoxide dismutase, even with microsomes depleted 10-fold of co-purifying enzyme by gel filtration, implies that superoxide was not involved in lipid peroxidation [29]. It also indicates that with the microsomal system equilibrium (3) is not easily displaced by superoxide dismutase. The lack of effect of catalase implies that peroxidation did not require H_2O_2. This, combined with inhibition by EDTA, appears to rule out initiation by the hydroxyl radical. In this respect, our conclusions differ from others [2, 11] who invoked the iron-catalysed Haber–Weiss reaction in adriamycin-dependent microsomal lipid peroxidation. We conclude that initiation is by a reduced iron complex, which can be formed directly by the microsomes, but much more efficiently with the adriamycin semiquinone as an intermediate. Whether this is simple a ferrous species, perhaps reacting with pre-existing lipid hydroperoxides to propagate the chain, or another type of iron complex, as suggested by others [30, 31] cannot be distinguished from our data. Differences in efficiency of iron chelates at catalysing lipid peroxidation have been observed before [11, 13, 32, 33], but are not clearly understood. They do not relate to reduction by the microsomes, since the EDTA, citrate, ADP and ATP complexes are reduced at similar rates (Vile and Winterbourn, in preparation). Neither is a site-specific mechanism the explanation, since ATP, citrate and EDTA all remove iron from microsomes yet ATP stimulates lipid peroxidation [33].

Our study, and the recent findings of Aust and coworkers [22] highlight the ease with which adriamycin, reduced by the microsomal reductase system, releases iron from ferritin, and allows it to participate in lipid peroxidation. We observed an appreciable increase in microsomal lipid peroxidation, only in the presence of adriamycin, with 10–20 μg/ml ferritin. Since normal heart contains 30–60 μg ferritin per gram [34], ferritin must be considered as a potential catalyst of *in vivo* adriamycin dependent peroxidation, and the pre-existence of low molecular weight iron complexes may not be necessary.

Although the heart is most susceptible to adriamycin toxicity, we, and others [2], have observed much more lipid peroxidation with rat liver microsomes than with heart microsomes. This does not necessarily apply for other species, however, since Mimnaugh *et al.* [2] saw much less of a difference in the mouse. Part of this can be attributed to differences in cytochrome P-450 reductase activity, but the high vitamin E level in rat heart may be another factor [2].

Previous studies have shown that adriamycin-dependent hydroxyl radical production is most efficient when the iron catalyst is reduced by the adriamycin semiquinone rather than by superoxide [15]. This is favoured by low pO_2. We have now shown that adriamycin-dependent peroxidation of microsomes, as well as liposomes [18] has a low pO_2 optimum. Even though normal intracellular O_2 concentrations can be as low as 5 mm Hg [35], few studies of adriamycin toxicity to cells or subcellular organelles have been carried out under these conditions. Our results suggest that much greater

evidence of oxidative reactions might be seen if physiological O_2 concentrations were employed, and they provide further support for such reactions contributing to the toxicity 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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