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LIPID PEROXIDATION AND POSSIBLE HYDROXYL RADICAL FORMATION STIMULATED BY THE SELF-REDUCTION OF A DOXORUBICIN–IRON (III) COMPLEX

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Abstract—In the presence of ferric ions, doxorubicin forms a complex which self-reduces the iron moiety to form a ferrous complex. This ferrous complex can generate active radicals able to degrade deoxyribose as well as form a species greatly stimulatory towards lipid peroxidation. Both reactions may explain the damage to different sites within the body associated with doxorubicin therapy.

Doxorubicin, an anthracycline antitumour antibiotic, is widely used in cancer therapy. The drug can intercalate cell DNA and so interfere with the processes of replication and RNA synthesis [1]. In addition, it has been observed to cause strand scission in the DNA molecule [2, 3] as well as a direct action on the cell membrane [4, 5]. Its mechanisms of cytotoxicity are therefore probably multiple in effect.

The clinical use of doxorubicin is limited by its toxicity, the most serious of which is a cumulative dose-dependent cardiomyopathy [6]. Doxorubicin interferes with electron transport in cardiac mitochondria [7] and stimulates peroxidation of cardiac lipids [8]. Doxorubicin and several other quinone-group containing drugs are readily reduced by the microsomal electron transport chain to form semiquinone radicals [9, 10]. Under partially anaerobic conditions, these semiquinone radicals can activate dioxygen and its reduction intermediates to form highly reactive radicals [11, 12] possibly hydroxyl radical, a species capable of abstracting hydrogen atoms from unsaturated fatty acids and causing strand scissions in the DNA molecule. However, although evidence strongly points to the hydroxyl radical as the species responsible for DNA damage [2, 3] it is difficult to demonstrate its contribution to the peroxidation of phospholipid membranes either in the presence or absence of doxorubicin [13, 14].

Recent work has shown that doxorubicin greatly stimulates ferric ion dependent lipid peroxidation [13, 15] and it has been proposed by Nakano and colleagues that this occurs by a self-reduction mechanism [16, 17]. Here, it is shown that during the self-reduction of a doxorubicin–ferric complex, ferrous species are formed which can activate dioxygen to generate reactive radicals capable of degrading deoxyribose. They also greatly stimulate lipid peroxidation by a mechanism previously shown not to involve the hydroxyl radical [13].

MATERIALS AND METHODS

Superoxide dismutase (bovine erythrocyte), catalase (bovine liver, thymol-free), albumin (fatty acid-free, human), lubrol PX and 2-deoxy-D-ribose were from Sigma Chemical Co. Ltd., (Poole, Dorset, U.K.). Desferrioxamine was from Ciba-Geigy and doxorubicin hydrochloride (adriamycin) was from Farmitalia Carlo Erba. All other chemicals were of the highest purity available and obtained from BDH Ltd. (Poole, Dorset, U.K.).

Phospholipid peroxidation. Bovine brain phospholipids were prepared as previously described [18]. Multilamellar liposomes were made containing 5 mg brain lipid per ml in 0.15 M NaCl, pH 7.4. Incubation mixtures contained 0.2 ml phosphate–saline buffer, pH 7.4 (0.024 M phosphate in 0.15 M NaCl), 0.2 ml of liposomal suspension and 20 μl of doxorubicin (1 mg/ml) dissolved in water. The peroxidation reaction was started by the addition of 0.1 ml of metal salt (1 mM) as ferric chloride or cupric chloride. After incubation at 37° for the appropriate times shown in Figs. 1 and 2, 0.5 ml of lubrol (1% w/v) was

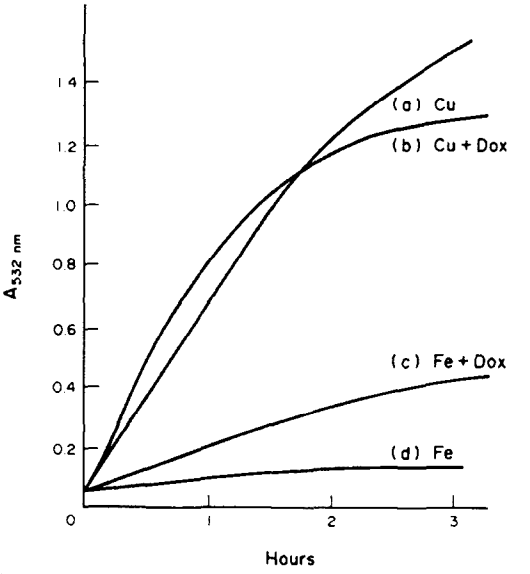

Fig. 1. Iron and copper salt stimulated peroxidation of bovine brain lipids measured as TBA reactivity at $A_{532\,nm}$. (a) Cupric salt only (0.19 mM); (b) cupric salt + doxorubicin (0.039 mg/ml); (c) ferric salt + doxorubicin (0.039 mg/ml); (d) ferric salt only (0.19 mM).

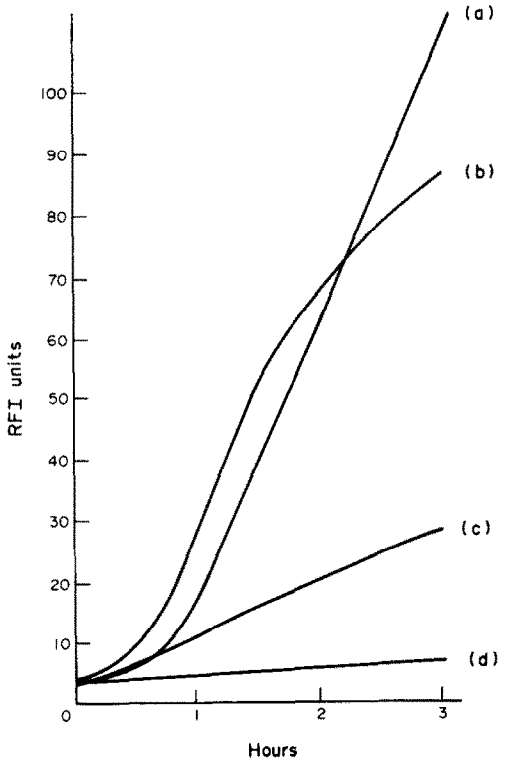

Fig. 2. Iron and copper salt stimulated peroxidation of bovine brain lipids measured as fluorescence units $Ex_{360\,nm}$, $Em_{430\,nm}$. (a) Cupric salt only (0.19 mM); (b) cupric salt + doxorubicin (0.039 mg/ml); (c) ferric salt + doxorubicin (0.039 mg/ml); (d) ferric salt only (0.19 mM).

added and the tube removed for measurement of lipid peroxidation.

Fluorescent measurements. Fluorescence formed in the reaction mixture during incubation was measured at excitation 360 nm and emission 430 nm and expressed as relative fluorescence intensity units (RFI) against a standard of tetraphenylbutadiene (10^{-7} M) using the instrument settings previously described [19].

Thiobarbituric acid (TBA) reactivity. After measurement of phospholipid fluorescence, 0.5 ml of 1% w/v thiobarbituric acid in 0.05 M NaOH was added together with 0.5 ml of 25% v/v HCl. The tubes were mixed and heated for 15 min at 100° to develop the colour. Resulting absorbance was read at 532 nm against appropriate blanks.

Deoxyribose degradation. Degradation of deoxyribose to TBA-reactive material was used as a method to detect radical damage [20, 21]. This was carried out in exactly the same way as for phospholipid peroxidation except 0.2 ml of 5 mM deoxyribose was substituted for the phospholipid and lubrol was not added at the end of the incubation period. Inhibitors and radical scavengers were added at the final reaction concentrations shown in Table 1 before addition of the metal salt. After incubation for 1 hr at 37°, 0.5 ml of the TBA reagent described above was added followed by 0.5 ml of 2.8% w/v trichloroacetic acid. The tubes were mixed and heated for 15 min at 100° to develop the colour.

Results of typical experiments are shown in Figs. 1 and 2 and in Table 1; these could be reproduced to within ±6%.

RESULTS

Cupric salts were considerably more stimulatory towards peroxidation of bovine brain lipids than were ferric ions (Figs. 1 and 2). These changes were reflected in both primary peroxidation products; the lipid peroxides detected non-specifically as TBA reactivity, and secondary carbonyl compounds detected as fluorescence. However, in the presence of the ferric salt, doxorubicin greatly stimulated peroxidation over the 3 hr period of incubation (Figs. 1 and 2). In contrast, cupric salts showed a variable response in the presence of doxorubicin changing from an increase in peroxidation during the early stages of incubation to a loss of stimulation after 2 hr (Figs. 1 and 2).

Deoxyribose degradation in the presence of metal salts has been shown to depend on the formation of active oxygen radicals [14, 21]. Here, as expected, neither cupric nor ferric ions alone could bring about deoxyribose degradation. However, in the presence of doxorubicin, ferric ions degraded deoxyribose (Table 1) whereas cupric ions did not (data not shown). The hydroxyl radical scavengers mannitol and thiourea substantially inhibited ferric ion–doxorubicin dependent damage to deoxyribose whereas the $OH^{\cdot}$ scavengers benzoate and formate did not (Table 1). The iron chelators desferrioxamine and EDTA both inhibited deoxyribose degradation (Table 1). Superoxide dismutase was not inhibitory whereas catalase was, suggesting that a reaction sequence similar to that summarized in Fig. 3 might be occurring. Caeruloplasmin was added to examine whether doxorubicin was reducing ferric ions to the ferrous state. This protein completely inhibited deoxyribose degradation (Table 1). Albumin and urea were added as controls for these reactions.

DISCUSSION

It has previously been shown that doxorubicin under partially anaerobic conditions is reduced, in a reaction catalysed by the enzyme xanthine oxidase, to form a semiquinone radical [11]. This radical together with traces of dioxygen and iron salts results in the formation of a species with reactivity similar to that of the hydroxyl radical. These radicals were detected by their ability to release TBA-reactive products from deoxyribose [20, 21].

Nakano and colleagues first proposed [16, 17] that a doxorubicin–iron(III) complex can, under aerobic conditions, by undergoing a self-reduction involving an intramolecular electron transfer, form an iron(II) complex (equations (1)–(3), Fig. 3). Neither ferric ions nor doxorubicin alone were able to generate a species capable of degrading deoxyribose. However, when present in the reaction together, deoxyribose was degraded by a reactive species with activity similar to the hydroxyl radical (Fig. 3).

Ferrous salts are known to be considerably more stimulatory towards lipid peroxidation than are ferric ions. Ferrous salts readily generate hydroxyl radicals in aqueous solution, and these have sufficient reactiv-

Table 1. Doxorubicin–iron(III) dependent damage to deoxyribose

	Deoxyribose degradation as TBA reactivity/hr			
	$A_{532\ nm}$	% Inhibition	$Ex_{532\ nm}$ $Em_{553\ nm}$ (RFI units)	% Inhibition
Doxorubicin only	0.106		35	
Doxorubicin + Fe(III), 0.16 mM	0.108		36	
Doxorubicin + deoxyribose	0.118		38	
Deoxyribose + Fe(III)	0.010		4	
Doxorubicin + deoxyribose + Fe(III) (control)	0.182		80	
Doxorubicin + superoxide dismutase, 0.08 mg/ml	0.184	0	86	0
Doxorubicin + superoxide dismutase, 0.016 mg/ml	0.184	0	83	0
Doxorubicin + catalase, 0.16 mg/ml	0.110	100	39	98
Doxorubicin + catalase, 0.02 mg/ml	0.112	100	36	100
Doxorubicin + albumin, 0.16 mg/ml	0.176	9	74	14
Doxorubicin + caeruloplasmin, 0.16 mg/ml	0.102	100	35	100
Doxorubicin + mannitol, 16 mM	0.130	81	36	100
Doxorubicin + thiourea, 1.6 mM	0.154	69	54	62
Doxorubicin + urea, 1.6 mM	0.179	5	83	0
Doxorubicin + benzoate, 16 mM	0.178	8	70	24
Doxorubicin + formate, 16 mM	0.183	2	73	17
Doxorubicin + desferrioxamine, 0.3 mM	0.137	70	37	100
Doxorubicin + EDTA, 0.3 mM	0.136	72	40	96

Results shown in Table 1 as % inhibition were calculated after subtraction of the doxorubicin deoxyribose blank value.

$$DXH_2 + Fe^{3+} \rightarrow DX^{\cdot}\text{–}Fe^{3+} + 2H^+ \quad (1)$$

$$DX^{\cdot}\text{–}Fe^{3+} \rightleftharpoons DX^{\cdot}\text{—}Fe^{2+} \rightleftharpoons Fe^{2+} + DX^{\cdot} \text{ (a semiquinone formed by oxidation of doxorubicin)} \quad (2)$$

$$Fe^{2+} \text{ (or possibly } DX^{\cdot}\text{–}Fe^{2+}) + O^2 \rightarrow Fe^{3+}\text{–}O_2^{\cdot -} \quad (3)$$

$$Fe^{3+}\text{–}O_2^{\cdot -} \rightarrow Fe^{3+} + O_2^{\cdot -} \quad (4)$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

$$Fe^{2+} \text{ (or possibly } DX^{\cdot}\text{–}Fe^{2+}) + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \text{ (or species with similar reactivity to } OH^{\cdot}) \quad (6)$$

$$DX^{\cdot} + H_2O_2 \xrightarrow[\text{catalyst}]{Fe} DX^+ + OH^{\cdot} + OH^- \text{ (or species with similar reactivity to } OH^{\cdot}) \quad (7)$$

Fig. 3. DXH_2 = doxorubicin. Reactions 1 and 2 summarize the self-reduction merchanism of a doxorubicin–ion (111) complex described by Sugioka *et al.* [16].

ity to initiate hydrogen abstraction from unsaturated lipids. However, so far it has not been possible to detect their contribution when peroxidation is stimulated by ferrous salts [14]. Similarly, no evidence was found for the participation of hydroxyl radicals in doxorubicin–iron(III) stimulated phospholipid peroxidation [13]. Ferric ion dependent peroxidation stimulated by doxorubicin might be attributed to either the self-reduction mechanism or to an increase in the solubility of iron(III) as the complex. Cupric ions and their complex with doxorubicin also greatly stimulated lipid peroxidation. The activity of this complex was, however, variable towards phospholipid peroxidation and did not damage deoxyribose suggesting it was not undergoing a self-reduction cycle analogous to iron.

Doxorubicin in the presence of a ferric salt will stimulate lipid peroxidation [13, 15] as well as the formation of an active radical capable of degrading deoxyribose. This reactive species has properties similar to that of the hydroxyl radical but may well not be as benzoate and formate did not protect. It can be formed under partially anaerobic conditions [11, 12] and as shown here under aerobic conditions too. Reactions involving both oxidation and reduction of the doxorubicin molecule, if they occur, *in vivo* would depend on the oxygen tension of the tissue as well as the availability of a suitable iron catalyst to complex with doxorubicin.

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