Inhibition by the protein ceruloplasmin of lipid peroxidation stimulated by an Fe^{3+}–ADP–adriamycin complex

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Received 20 October 1983; revised version received 5 December 1983

Self-reduction of an Fe^{3+}–ADP–adriamycin complex under anaerobic conditions and reduction of ferricytochrome *c* by the complex under aerobic conditions were strongly inhibited by ceruloplasmin, but not by superoxide dismutase or albumin at the same protein concentration. Ceruloplasmin, a protein with ferroxidase activity, is able to catalyse oxidation of Fe^{2+} to the ferric state. The inhibitory activity of ceruloplasmin towards reactions stimulated by the complex suggests that Fe^{2+} is formed during the self-reduction process. As expected, the Fe^{3+}–ADP–adriamycin complex stimulated lipid peroxidation in which the Fe^{2+} moiety was implicated. This stimulation was again effectively prevented by ceruloplasmin but not by superoxide dismutase.

Ceruloplasmin *Superoxide dismutase* *Adriamycin* *Ferroxidase activity*
Lipid peroxidation *Oxygen radical*

1. INTRODUCTION

Ceruloplasmin, the copper-containing protein of extracellular fluids, has been shown to have important antioxidant properties towards peroxidising lipids [1–3]. These activities can be mainly ascribed to its known ferroxidase activity resulting in a catalysed oxidation of Fe^{2+} to the ferric state as well as an ability to inactivate copper ions [4].

The anthracycline antitumour antibiotic adriamycin has been shown, in vivo, to induce peroxidation of cardiac lipids [5]. Studies in vitro, however, have shown that adriamycin itself does not stimulate lipid peroxidation until it is first co-ordinated with Fe^{3+} [6]. Authors in [6,7] proposed a mechanism for adriamycin-induced lipid peroxidation involving an Fe^{3+}–chelated adriamycin complex in which the Fe^{3+} moiety of the complex could undergo self-reduction or enzymic reduction [6,7].

If ceruloplasmin can catalyse oxidation of the Fe^{2+} moiety in the chelated complex, then adriamycin–iron-stimulated lipid peroxidation should be strongly inhibited by its ferroxidase activity. This work was undertaken to examine this proposal.

2. MATERIALS AND METHODS

2.1. *Reagents*

Superoxide dismutase (spec. act. 2900 units/mg), albumin (bovine, fatty acid-free), cytochrome *c* (horse heart, type VI) and ceruloplasmin (human, type III) were obtained from Sigma. Adriamycin (doxorubicin hydrochloride) was a gift from Kyowa Hakko Co. Ferrocytochrome *c* was prepared by dithionite reduction of ferricytochrome *c* and passed through a Sephadex G-25 column (1.5 × 12 cm) which had been equilibrated with 10 mM Tris–HCl buffer at pH 7.5, using the same buffer to remove excess dithionite. During and after elution of ferrocytochrome *c*, the buffer and eluate were gassed with nitrogen.

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00145793/84/$3.00

2.2. *Phospholipid*

Microsomal phospholipid was extracted from rat liver microsomes using chloroform–methanol [8] and was freed from contaminating lipids (free fatty acids, cholesterol and its ester) by a silicic acid column chromatography [9].

2.3. *Incubation experiments*

Components of the reaction mixture, such as phospholipid (as liposomes) and the Fe^{3+}–ADP complex, were prepared just before use as in [7].

Components of the co-ordination complex, Fe^{3+}–ADP or Fe^{3+}–ADP–adriamycin were expressed in molar concentrations. The Fe^{3+}–ADP–adriamycin complex prepared under our experimental conditions had a co-ordination ratio of about 2 for the adriamycin (ligand) to Fe^{3+} (co-ordination metal) [6]. A Thunberg-type cell with a sidearm was employed for anaerobic conditions. The contents (0.2 mM adriamycin and enzyme – when added – in 0.1 M Tris–HCl buffer, pH 7.5) of the cell were rapidly frozen using solid CO_2, followed by evacuation at 8 mm Hg at room temperature, until the contents were completely thawed. This was repeated 8 times in all. The reaction was initiated by mixing the Fe^{3+}–ADP complex held in the sidearm with the cell contents.

For all incubation experiments, the reaction was initiated by the addition of Fe^{3+}–ADP complex and continued at 37°C for the times indicated.

2.4. *Assays*

Self-reduction of the Fe^{3+}–ADP–adriamycin complex under anaerobic conditions was measured by the decrease in absorbance at 602 nm [6,7].

Oxygen consumption was measured in an 'Instech' oxygenometer (Model 102) with a Clark-type electrode [7].

Oxidation and reduction of cytochrome *c* were monitored by changes in absorbance at 550 nm [7]. Fatty acid composition was determined by a modification of the method in [10] using a Shimadzu gas chromatograph (Model GC-4CM).

3. RESULTS

3.1. *Inhibition of self-reduction of the Fe^{3+}–ADP–adriamycin complex by ceruloplasmin*

When 0.2 mM adriamycin in 0.1 Tris–HCl buffer (pH 7.5) was mixed with the Fe^{3+} (0.1 mM)–ADP (1.67 mM) complex under both anaerobic and aerobic conditions it showed a characteristic absorption band at 602 nm [7]. Under anaerobic conditions the intensity of absorbance then decreased at 602 nm with increasing time, suggesting the self-reduction of the Fe^{3+}–ADP–adriamycin complex in which Fe^{3+} was converted to Fe^{2+} (fig.1, curve 1). Superoxide dismutase (0.05 mg/ml, 1.6×10^{-6} M) did not alter the original decay curve of the Fe^{3+}–ADP–adriamycin complex (fig.1, curve 2). The self-reduction process was biphasic, showing an initial slow reaction followed by a faster phase. The first response may represent the initiation of intramolecular electron transfer resulting in reduction of the iron moiety. Ceruloplasmin (0.05 mg/ml, 3.3×10^{-7} M) caused almost complete inhibition of the initial phase (fig.1, curve 3). However, inhibition did not last long, probably because of loss of ferroxidase activity under anaerobic conditions [11]. A further consideration for the reaction response is the stoichiometry between Fe^{2+} in the complex and copper ions in the enzyme. Ceruloplasmin (with 6–7 copper

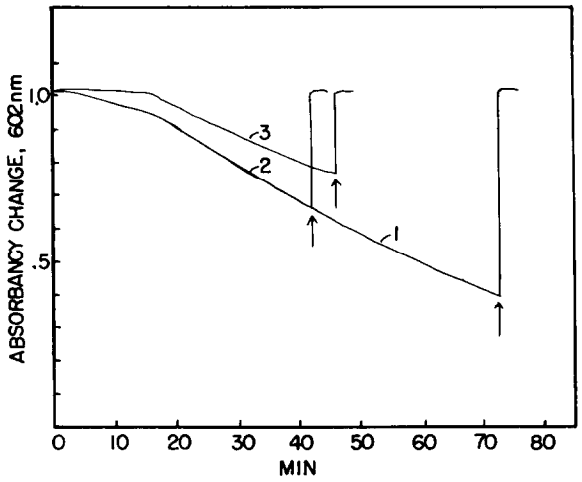

Fig.1. Decrease of a characteristic absorption band (at 602 nm) for the Fe^{3+}–ADP–adriamycin complex under anaerobic conditions. The reaction mixtures and experimental conditions are described in section 2. 1, Fe^{3+}–adriamycin complex; 2, Fe^{3+}–ADP–adriamycin complex plus superoxide dismutase; 3, Fe^{3+}–ADP–adriamycin complex plus ceruloplasmin. Arrow indicates the time at which air was bubbled into the reaction mixture.

atoms/molecule) would, in this reaction, show a copper concentration corresponding to about 1/13 of the iron present. Little or no inhibition of the decrease in absorbance at 602 nm was observed with heat-denatured ceruloplasmin (not shown). In all experiments, intensity at A_{602nm} returned to the original level following re-oxygenation (fig.1).

3.2. *Inhibition by ceruloplasmin of cytochrome c reduction by the Fe^{3+}–ADP–adriamycin complex*

Ferricytochrome *c* was reduced to ferrocytochrome *c* by the Fe^{3+}–ADP–adriamycin complex under both anaerobic and aerobic conditions, suggesting an electron transfer from the Fe^{2+}–ADP–adriamycin complex (or a structurally related complex) produced by its self-reduction [7]. If oxidation of the Fe^{2+}–ADP–adriamycin complex is catalysed by ceruloplasmin, the reduction of cytochrome *c* by the Fe^{3+}–ADP–adriamycin complex should also be inhibited by ceruloplasmin. As shown in fig.2A, autooxidation of ferrocytochrome *c* under aerobic conditions was promoted by native as well as heat-denatured ceruloplasmin. This may be attributed to non-specific activity associated with commercially obtained preparations of ceruloplasmin. As shown in fig.2B, intact ceruloplasmin caused a strong inhibition under aerobic conditions of the Fe^{3+}–ADP–adriamycin-induced cytochrome *c* reduction, while heat-denatured ceruloplasmin showed only a slight inhibitory effect. Little or no inhibition was observed following the addition of albumin or superoxide dismutase at the same protein concentration (0.05 mg/ml) as that for ceruloplasmin.

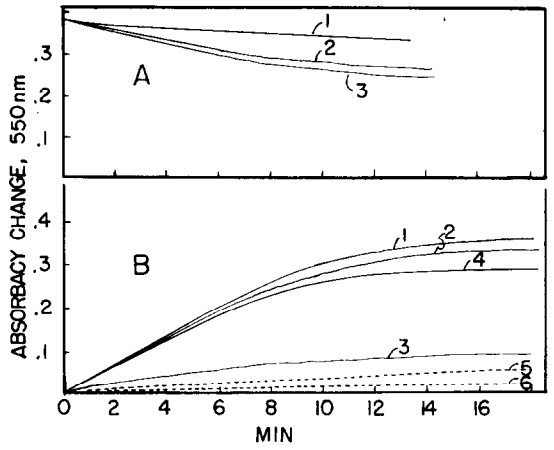

Fig.2. Effect of various proteins on the autooxidation of ferrocytochrome *c* (A), and on cytochrome *c* reduction by the Fe^{3+}–ADP-complex (B), both in air. (A) The reaction mixture contained 0.018 mM ferrocytochrome *c* (freshly prepared) and protein at 0.05 mg/ml (or none) in 0.1 M Tris–HCl buffer (pH 7.5). 1, ferrocytochrome *c* (basal), basal plus superoxide dismutase or basal plus albumin; 2, basal plus ceruloplasmin; 3, basal plus heat-denatured ceruloplasmin. (B) The reaction mixture contained Fe^{3+} (0.1 mM)–ADP (1.67 mM)–adriamycin (0.2 mM), 0.018 mM ferricytochrome *c* and protein at 0.05 mg/ml (or none) in 0.1 M Tris–HCl buffer (pH 7.5). 1, ferricytochrome *c* plus Fe^{3+}–ADP–adriamycin complex (basal); 2, basal plus superoxide dismutase; 3, basal plus ceruloplasmin; 4, basal plus heat-denatured ceruloplasmin; 5, basal minus Fe^{3+}–ADP (control); 6, control plus ceruloplasmin.

3.3. *Inhibition by ceruloplasmin of Fe^{3+}–ADP–adriamycin stimulated lipid peroxidation*

Peroxidation of phospholipid liposomes in 0.1 M Tris–HCl buffer (pH 7.5) was monitored by an increase in oxygen consumption and changes in the fatty acid composition of the phospholipids. Oxygen consumption was markedly enhanced by

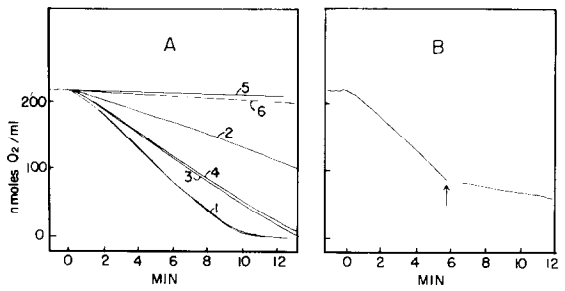

Fig.3. Oxygen consumption in the systems containing liposomes, the Fe^{3+}–ADP–adriamycin complex and protein (or none). (A) The reaction mixture contained liposomes (0.85 μmol/ml), Fe^{3+} (0.1 mM)–ADP (1.67 mM)–adriamycin (0.2 mM) complex and protein (0.05 mg/ml) or none in 0.1 M Tris–HCl buffer (pH 7.5). 1, Fe^{3+}–ADP–adriamycin and liposomes (basal) or basal plus albumin; 2, basal plus ceruloplasmin; 3, basal plus heat-denatured ceruloplasmin; 4, basal plus superoxide dismutase; 5, basal minus the Fe^{3+}–ADP–adriamycin complex; 6, basal minus liposomes. (B) The reaction mixture was the same as basal (1) in A. Arrow indicates the time at which ceruloplasmin (0.05 mg/ml) was added.

Table 1

Effect of ceruloplasmin on phospholipid fatty acid composition after peroxidation stimulated by an Fe^{3+}–ADP–adriamycin complex

Incubation system[a]	Fatty acid composition[b] (mol/100 mol palmitic acid)					
	16:0	18:0	18:1	18:2	20:4	22:6
S1 Suspension of phospholipid micelles	100	71	33	57	100	24
S2 Basal (after perodixation)	100	72	33	51	67	12
S3 S2 + ceruloplasmin	100	72	33	55	84	18
S4 S2 + heat-denatured ceruloplasmin	100	70	34	54	75	14
S5 S2 + superoxide dismutase	100	73	33	53	76	16
S6 S2 + albumin	100	72	33	51	67	13

[a] The reaction mixtures and incubation conditions were the same as those described in the legend to fig.3. Samples were incubated for 15 min

[b] Results were expressed as means of 3 experiments which were reproducible to ± 5%

the addition of the Fe^{3+}–ADP complex to the liposomal system containing 0.2 mM adriamycin. Oxygen consumption was substantially inhibited by the addition of ceruloplasmin (fig.3A) either before or during the reaction (fig.3B). It was also inhibited, but to a much smaller extent, by heat-denatured ceruloplasmin and native superoxide dismutase (fig.3A). Albumin, at the same protein concentration as ceruloplasmin, did not affect oxygen consumption. Under similar conditions, destruction of polyunsaturated fatty acids, especially arachidonic (20:4) and docosahexaenoic (22:6) acids, induced by the addition of Fe^{3+}–ADP–adriamycin was effectively prevented by ceruloplasmin but not by heat-denatured ceruloplasmin or intact superoxide dismutase (table 1).

4. DISCUSSION

It has been reported previously [6,7] that an Fe^{3+}–ADP–adriamycin complex stimulates the peroxidation of phospholipid liposomes without the need to add a reducing system (e.g., NADH-dependent cytochrome P-450 reductase). Furthermore, it has been suggested that the co-ordination complex is aerobically converted to a perferryl ion-complex, via the Fe^{2+}–ADP–adriamycin radical, which stimulates the observed lipid peroxidation [7]. However, the Fe^{3+}–ADP–adriamycin complex which is formed in Tris–HCl buffer can be dissociated into Fe^{3+}–ADP (or Fe^{3+}–ADP–phosphate) and adriamycin when present in a phosphate buffer. This has been observed to decrease stimulation of lipid peroxidation [12]. In addition, a simple Fe^{3+}–adriamycin complex has also been observed to stimulate lipid peroxidation [6,13].

Here, the strong inhibitory effect of ceruloplasmin towards the spontaneous decrease in absorbance at 602 nm (due to the self-reduction of the Fe^{3+}–ADP–adriamycin complex) under anaerobic conditions and also to reduction of cytochrome *c* by the Fe^{3+}–ADP–adriamycin complex under aerobic conditions has been demonstrated. This clearly indicates that the reduced iron moiety in the complex, which can be produced both by self-reduction and in the presence of an electron acceptor, is re-oxidised in a reaction catalysed by ceruloplasmin. Furthermore, ceruloplasmin offered a substantial protection against Fe^{3+}–ADP–adriamycin-stimulated lipid peroxidation as monitored by O_2 uptake and estimation of changes in liposomal phospholipid fatty acids. This indicates that a species of Fe^{2+} (possibly the perferryl ion derived from Fe^{2+} co-ordinated with ADP and adriamycin) plays an important role in stimulating lipid peroxidation. In addition, a simple iron salt and iron chelate, Fe^{2+}–ADP, may also contribute.

The concentrations of superoxide dismutase used in the present experiments were far greater

than those required for its catalytic activity. Therefore, its inhibitory activity towards lipid peroxidation may not necessarily be of a truly enzymic nature since superoxide dismutase at 5×10^{-7} M had no effect on lipid peroxidation stimulated by the Fe^{3+}–ADP–adriamycin complex with [7] or without NADH-cytochrome P-450 reductase (not shown).

From the present results and those obtained previously [7] we can tentatively propose the following scheme:

$$Fe^{3+}\text{–ADP–ADM} \rightleftharpoons Fe^{2+}\text{–ADP–ADM}^{+} \rightleftharpoons Fe^{2+} + \text{ADP} + \text{ADM}^{+} \quad (1)$$

$$Fe^{2+}\text{–ADP–ADM}^{+} + O_2 \longrightarrow O_2^{-}..Fe^{3+}\text{–ADP–ADM}^{+} \quad (2)$$

$$O_2^{-}..Fe^{3+}\text{–ADP–ADM}^{+} \longrightarrow \text{stimulates lipid peroxidation} \quad (3)$$

$$Fe^{2+}\text{–ADP–ADM}^{+} + \text{Cyt}(Fe^{3+}) \longrightarrow Fe^{3+}\text{–ADP–ADM}^{+} + \text{Cyt}(Fe^{2+}) \quad (4)$$

$$Fe^{2+}\text{–ADP–ADM}^{+} + \text{E}(Cu^{2+}) \longrightarrow Fe^{3+}\text{–ADP–ADM}^{+} + \text{E}(Cu^{+}) \quad (5)$$

where $O_2^{-}..Fe^{3+}$–ADP–ADM^{+}, Cyt and E represent the perferryl ion complex, cytochrome *c* and ceruloplasmin, respectively. Reaction 5 should be considerably faster than reactions 2 and 4.

In conclusion, an iron–ADP–adriamycin complex can undergo a self-reduction to produce active oxygen species able to stimulate lipid peroxidation. The reactivity of this species is dependent on the Fe^{2+} moiety which can be oxidised catalytically by the ferroxidase activity of the protein ceruloplasmin.

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