Transfer of ferritin-bound iron to adriamycin

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Interactions of adriamycin with ferritin-bound iron have been investigated. It is demonstrated (i) that adriamycin stimulates an iron-dependent lipid peroxidation in submitochondrial particles in the presence of ferritin, and (ii) that incubation of adriamycin with ferritin results in a slow transfer of iron to adriamycin with formation of an adriamycin–iron complex. The results are discussed in relation to the possible role for intracellular iron in adriamycin toxicity.

Ferritin *Adriamycin–Fe^{3+} complex* *Lipid peroxidation* *Submitochondrial particle*

1. INTRODUCTION

Generation of reactive free radicals has been implicated as one of the factors that may contribute to the development of the special cardiotoxicity of adriamycin [1–4]. It is well established from in vitro studies that adriamycin binds Fe^{3+} with considerable affinity [5,6], forming complexes which promote free radical-related damage to cell constituents such as DNA [7] and membrane phospholipids [8,9]. It is, however, not clear whether toxic adriamycin–iron complexes can form in vivo. Ferritin, the major storage of iron in cells [10,11], is one possible intracellular source of iron for formation of adriamycin–iron complexes. Heart tissue contains 30–60 μg ferritin protein/g [10]. The ferritin molecule consists of a crystalline core of ferric oxyhydroxide, containing up to 4500 iron atoms, surrounded by a spherical protein shell, 150 Å in diameter and about 25 Å thick. Here we examine whether the affinity of adriamycin for iron is sufficient to allow transfer of ferritin-bound iron to adriamycin.

2. MATERIALS AND METHODS

2.1. *Conditions for determining transfer of ferritin iron to adriamycin*

Incubations of ferritin with adriamycin were carried out in 150 mM KCl, 50 mM Tris–HCl buffer (pH 7.4) at 30°C in the dark. Samples were removed from the incubation mixtures at intervals and adriamycin–Fe^{3+} complex was determined spectrophotometrically at 600 nm in 1 cm light-path cuvettes. The absorptions at 600 nm of adriamycin and ferritin, incubated separately, were subtracted from the measurements.

2.2. *Determination of ferritin iron released by ADP*

Release of ferritin iron in ADP containing incubation buffer without adriamycin was assayed by adding 10 μl 10 mM bathophenanthroline disulphonate and 10 μl 10 mM ascorbic acid to 1 ml samples. The concentration of 'free' iron was calculated from the increase in absorbance at 534 nm obtained after 2 min using ϵ = 22140 $M^{-1} \cdot cm^{-1}$ [12].

2.3. *Other methods*

Submitochondrial particles were prepared from pig heart mitochondria as described in [9] but using 250 mM sucrose in the buffers instead of 150 mM KCl.

Measurements of NADH oxidase activity in submitochondrial particles incubated together with ferritin and adriamycin, protein determination, extraction of particle phospholipid, fatty acid ana-

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lysis by GLC and determination of adriamycin and $FeCl_3$ stock solutions were carried out as described previously [9]. Total ferritin iron was determined by the method described in [13].

Stability of adriamycin during incubation was assessed by thin-layer chromatography (TLC) on 0.25 mm silica gel plates (Merck, Darmstadt, FRG) using the solvent chloroform/methanol/acetic acid (46:15:6, v/v).

2.4. *Materials*

Ferritin (from horse spleen, cadmium-free and 3 × crystallized) and ADP were from Boehringer (Mannheim, FRG). Bathophenanthroline disulphonate was from Sigma (St. Louis, USA). Adriamycin/HCl was a gift from Farmitalia Carlo Erba (Milano, Italy).

Buffer solutions were prepared from analytical grade reagents the day before use with water purified by ion exchange in a Millipore system (Millipore).

3. RESULTS

Adriamycin–iron complexes support peroxidative damage to respiratory chain activity and phospholipids in submitochondrial particles [9, 14]. When submitochondrial particles were incubated with horse spleen ferritin (50 μg/ml corresponding to 196 μM ferritin iron) about 30% of their NADH oxidase activity was lost during a 3-h period. Inclusion of adriamycin into the incubation mixtures caused a drastic increase in the rate of inactivation as shown in fig.1. Also, accelerated peroxidative breakdown of polyunsaturated fatty acid moieties of phospholipids in the particles incubated with ferritin was measured upon addition of adriamycin to the incubation mixtures (table 1). Both NADH oxidase activity and phospholipids were protected in the presence of 1 mM EDTA.

Spectrophotometric determination of adriamycin–Fe^{3+} complex formed during the incubation of ferritin with adriamycin was carried out using absorption of the complex at 600 nm [15]. 600 nm absorbing material was found to develop with time in incubation mixtures containing ferritin (50 μg/ml) together with adriamycin (25–200 μM) (not shown). However, a visible colloid appeared during the incubations and it could not be excluded

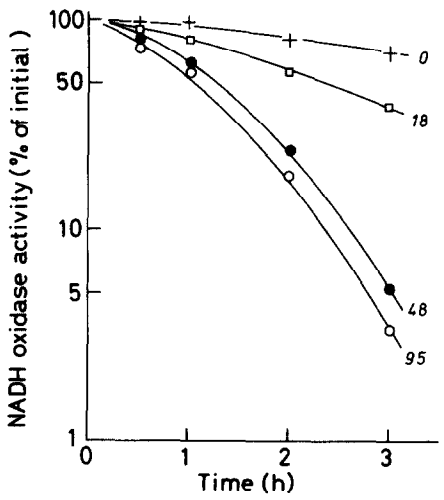

Fig. 1. Inhibition of NADH oxidase activity in pig heart submitochondrial particles by adriamycin during incubation with ferritin. Particle protein (94 μg/ml), ferritin (50 μg/ml) and adriamycin (0–95 μM).

Table 1

Loss in phospholipid arachidonic acid and NADH oxidase activity of submitochondrial particles incubated with adriamycin and ferritin

Incubation conditions	Arachidonic acid in particle lipid (% of control)	NADH oxidase activity (% of control)
Adriamycin	91.3	89.4
Ferritin	76.5	69.1
Adriamycin + ferritin	20.2	4.7
+ EDTA (1 mM)	72.1	92.0

Particle protein (86 μg/ml), adriamycin (50 μM), ferritin (50 μg/ml). Incubation period was 3 h and control particles were incubated in the absence of adriamycin and ferritin

that part of the measured increase in 600 nm absorbance was due to light scattering in the cuvettes. We found that colloid formation could be prevented by 1 mM ADP and the incubations of ferritin with adriamycin were therefore repeated in ADP-containing buffer. Results are given in fig.2. It is seen that 600 nm absorbing material also formed under these conditions and at a rate that increased with adriamycin concentration. No increase in 600 nm absorbance was measured when 1 mM EDTA

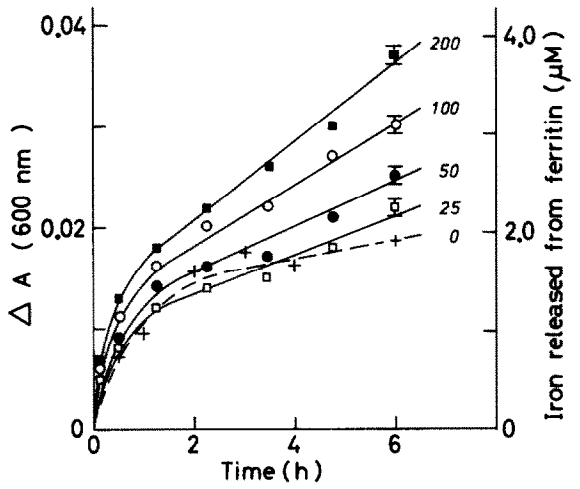

Fig. 2. Iron transfer from ferritin to adriamycin. Ferritin (50 μg/ml) and adriamycin (0–200 μM) were incubated in buffer containing 1 mM ADP. Values represent the mean of 3 separate experiments. Bars are SE. (– – –), release of ferritin iron in the absence of adriamycin.

was added to the incubation mixtures or when adriamycin and ferritin were incubated separately (not shown).

Addition of Fe^{3+} (as a freshly prepared 1 mM $FeCl_3$ in 1 mM HCl) to adriamycin in ADP-containing buffer was found to result in a rapid formation of adriamycin-Fe^{3+} complex (within less than 1 min) and the obtained 600 nm absorbance was a linear function of the Fe^{3+} concentration up to 10 μM. A molar absorption coefficient of 9700 $M^{-1} \cdot cm^{-1}$ was calculated, which is close to the value of 11 400 found by authors in [15] in buffer containing acetohydroxamic acid. The absorbance measurements in fig.2 could therefore be taken as a direct measure for the concentration of iron released from ferritin as given by the second ordinate. The 4 μM iron released during the 6-h incubation period corresponds to 2% of the total iron in the ferritin.

ADP alone can release ferritin-bound iron and the progress curves for formation of adriamycin-Fe^{3+} complex in fig.2 can be expected to express the sum of iron release by ADP and adriamycin. The contribution from ADP was estimated using the iron, chelator bathophenanthroline disulphonate during incubation of ferritin in ADP containing buffer without adriamycin (see section 2.2.). The results are given by the dotted line in fig.2.

4. DISCUSSION

We conclude from the above results that ferritin-bound iron can be transferred to adriamycin and that the adriamycin-iron complexes formed support peroxidative damage to membrane lipids and enzyme activities.

It is well documented that ferritin iron can be released by iron chelators [16,17] and by reducing agents such as ascorbate, thiols and dihydroflavins (reduced riboflavin, $FMNH_2$, $FMNH_2$) [18–22]. It has also been demonstrated [23,24], that ferritin in the presence of ascorbate can serve as source of iron in lipid peroxidation reactions. Adriamycin has iron binding as well as reducing properties, possibly owing to its quinone-hydroquinone structure [25], and the size of the molecule is probably small enough (similar to that of $FADH_2$) to have direct access to the iron core of ferritin through the pores of the protein shell. The biphasic time course for formation of adriamycin-Fe^{3+} complex may be explained by the presence of a small fraction of iron contained in the ferritin molecule in a loosely bound form [20].

Cells, also myocardial cells, are able to take up large amounts of adriamycin [26–28], but little information is available on its intracellular routing. The present data suggest that adriamycin-iron complexes could form, resulting in damage to cell components sensitive to free radical attack. Studies on cultured cells with a varied iron-loading and ferritin content [29,30] could contribute with valuable results in regard to the possible role for intracellular iron in adriamycin toxicity.

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