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INACTIVATION OF CYTOCHROME *c* OXIDASE ACTIVITY IN MITOCHONDRIAL MEMBRANES DURING REDOX CYCLING OF DOXORUBICIN

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Abstract—Interactions of doxorubicin (DX) with the cardiolipin-dependent cytochrome *c* oxidase have been examined by using pig heart submitochondrial particles (SMP). A progressive and irreversible loss of oxidase activity is demonstrated in 2 hr incubations of the SMP with 10–100 μM DX in air-equilibrated medium with excess NADH to support redox-cycling of the drug. This oxidative mechanism for oxidase inactivation occurs in connection with a peroxidation process in the bulk membrane lipid, and is independent on turnover of the enzyme. It is related in a complex manner to the electron flux in the respiratory chain with antioxidant properties, and is maximal at the high reduction level of respiratory chain Complex I obtained in the presence of rotenone. Reduction of DX *per se* plays a minor role, and trace concentrations of chelatable metal ions (iron) are required to catalyse the reaction. Iron in the iron storage protein ferritin is released by DX, and at physiological low O_2 concentrations ($[O_2] < 20$ μM), this iron is a better promoter of oxidase inactivation than is endogenous iron in the SMP. Kinetic analysis of inactivation data indicates the interaction of DX with low affinity (K_m 35–55 μM) binding sites in the SMP membranes. Overall, the results point to the possible role of ferritin–iron in the mechanism of DX mitochondrial toxicity and argue against site specific effects of the DX-reduction/oxidation cycle on the cytochrome *c* oxidase or on its essential phospholipid (cardiolipin) environment.

The anthracycline aminoglycoside doxorubicin (Adriamycin®) (DX)* forms a tight complex with the double charged anionic phospholipid cardiolipin contained in the inner mitochondrial membranes [1–3]. This specific drug–lipid interaction has been suggested to play a key role in the mitochondrial toxicity of DX [1, 4, 5]. Membrane binding of DX has significant effects on the lipid structural state [6–8], and there is evidence that relates DX-induced inhibition of the mitochondrial cytochome *c* oxidase (Ferrocytochrome *c*: oxygen oxidoreductase; EC 1.9.3.1) to a phase separation of the cardiolipin molecules required for optimal activity of the enzyme [5, 9, 10]. Chemical reactions must, however, also be taken into account when DX interacts with mitochondria carrying out substrate oxidation through the respiratory chain. The quinone functional group of the anthracycline is then reduced in the first section of the respiratory chain to form the DX-semiquinone free radical [11, 12]. Subsequent reoxidation of the semiquinone takes place either via reactions with respiratory chain components in an oxidized state (cytochrome *c* and Complex III) [13, 14] or with molecular oxygen to yield superoxide radicals and hydrogen peroxide [15, 16]. The purpose of the study was to determine the ability of DX to inhibit the mitochondrial cytochrome *c* oxidase during this cyclic reduction–oxidation process.

* Abbreviations: Buffer I, 150 mM KCl, 10 mM Na/Hepes (pH 7.4); DX, doxorubicin; DETAPAC, diethylenepentaacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *N*-acetylDX, *N*-acetyldoxorubicin; NHS-DX, *N*-hydroxysuccinimide ester derivative of doxorubicin; PE, phosphatidylethanolamine; SMP, submitochondrial particles; TLC, thin-layer chromatography; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid.

MATERIALS AND METHODS

Materials. NADH (grade I, 100%), superoxide dismutase (from bovine erythrocytes, 5000 units/mg), catalase (from beef liver, 65,000 units/mg), xanthine oxidase (from cows milk, 1 unit/mg), and ferritin (from horse spleen) were obtained from Boehringer (Mannheim, F.R.G.). Xanthine oxidase and catalase were filtered on a Sephadex G-25 column to remove ammonium sulfate, EDTA and other contaminants prior to use. Doxorubicin/HCl, cytochrome *c* (from horse heart, Type VI), human serum transferrin (98%, substantially iron-free), bathophenanthrolinedisulfonic acid (4,7-diphenyl-1,10-phenanthrolinedisulfonic acid), bathocuproinedisulfonic acid (2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid), *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, rotenone, antimycin A, and menadione were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The cross-linker *bis*(sulfosuccinimidyl) suberate (BS^3) was from Pierce Chemical Co. (Rockford, IL, U.S.A.), and desferrioxamine B methanesulfate (Desferal) from Ciba Geigy (Copenhagen, Denmark).

Submitochondrial particles (SMP) were made by sonication of pig heart mitochondria in 0.25 M sucrose containing 2 mM EDTA (pH 8.5) [17]. The particles were washed twice by centrifugation at 100,000 *g* for 30 min in 0.25 M sucrose, 10 mM Tris–HCl buffer (pH 7.4), frozen in liquid nitrogen at a

concentration of 40–50 mg protein/mL, and stored at −80°.

Cardiolipin (diphosphatidylglycerol) was isolated as the sodium salt from total lipid extracts of mitochondria and stored in ethanol at −20° under nitrogen. The fatty acid content (mol %) was 0.4% 16:0, 0.7% 18:0, 9.6% 18:1, 86.9% 18:2, and 0.7% 20:4, and the peroxide content <2 nmol/mg lipid when determined with the iodometric assay [18].

Conditions for incubation of SMP. All the incubations of SMP were carried out in the dark at 30° in 150 mM KCl, 10 mM Na/Hepes buffer (pH 7.4) (Buffer I) with slow stirring of the particles (0.10 mg protein/mL). Stock solutions of 1.5 M KCl and 0.1 M Hepes were passed through a Chelex 100 resin (100–200 mesh, Bio-Rad Laboratories, Richmond, CA, U.S.A.) just before use to remove contaminating metal ions. Incubation buffer with 100 μM DX and 200 μM NADH contained 0.05–0.15 μM iron and copper. Stock solutions of DX/HCl (5 mM) were in distilled water and NADH (0.5–20 mM) in incubation buffer. Rotenone (1.25 mM), antimycin A (0.1 mM), and menadione (2–20 mM) were in ethanol. The oxygen concentrations in the SMP suspensions were measured with a Clark-type oxygen electrode, and adjusted by equilibration with air, oxygen/nitrogen mixtures, or nitrogen passed through 1% pyrogallol in 0.1 M NaOH.

In experiments examining the involvement of metal ions in oxidase inactivation, SMP suspensions in Buffer I (5 mg protein/mL) were treated with 10 mM EDTA for 1 hr at 0° followed by filtration on Sepharose CL-2B to remove contaminating ferritin [19] and loosely bound metal ions from the particles [20]. In selected experiments, DX and NADH stock solutions were dialysed against apotransferrin to minimize iron contamination [21]. Ferritin was treated with EDTA and passed through a Sephadex G-25 column [20] just before addition to the incubation mixtures. Metal ions dissolved in 1 mM HCl were added together with DX.

Assay of oxidase activities and DX redox-cycling. Samples (10–50 μL) were removed from the incubation mixtures for assay of oxidase activities. Cytochrome *c* oxidase activity was assayed spectrophotometrically by measuring the initial rate of oxidation of 50 μM ferrocytochrome *c* followed at 550 nm (Buffer I, 1 mL final volume, 30°). Polarographic assay was done by measuring oxygen uptake with an oxygen electrode in 10 mM Tris–HCl buffer (pH 7.4) with 0.5 mM TMPD plus 2.5 mM ascorbate. NADH oxidase activity was measured by the initial rate of oxidation of 100 μM NADH followed at 340 nm (Buffer I, 30°). Data were analysed statistically by Student's *t*-test.

Reduction of DX and superoxide radical generation in incubation mixtures with SMP was estimated as the rate of superoxide dismutase-inhibitable reduction of acetylated ferricytochrome *c* (50 μM) followed at 550 nm [22]. Xanthine oxidase-catalysed superoxide formation in the absence of SMP was followed by reduction of ferricytochrome *c* (50 μM) in air-equilibrated Buffer I with 0.4 mM xanthine and catalase (100 μg/mL) [23]. Xanthine oxidation was followed at 295 nm [23].

Protein and lipid analysis. SMP-protein was measured according to Lowry *et al.* [24] with bovine serum albumin as the standard. Following incubation of SMP, the particles were sedimented by centrifugation at 200,000 *g* for 60 min (4°) in the presence of 10 mM EDTA and 10 μM of the antioxidant 2,6-bis(*tert*-butyl)-4-hydroxytoluene. Heme *a* was determined from dithionite-reduced minus air-oxidized difference spectra using the wavelength pair 605–630 nm [25]. Oxidase reactivation experiments were performed in which the SMP particles were dissolved in 1% (w/v) Triton X-100 with 100 μM of mitochondrial cardiolipin prior to the assay in 0.5% (w/v) Tween 80 [26]. Extractions of total SMP-lipid into chloroform/methanol, separation of phospholipids by TLC, and fatty acid analysis by gas–liquid chromatography, were carried out as previously described [27]. Lipid phosphorus was determined according to Bartlett [28].

Iron and copper determinations. The total iron and copper contents of buffer solutions, DX and NADH were determined spectrophotometrically with bathophenanthrolinedisulfonate and bathocuproinedisulfonate after wet ashing of samples with H_2SO_4 and H_2O_2 [29]. Total SMP-iron not associated to heme or iron–sulfur proteins was estimated from $\Delta(A_{540\,nm}-A_{575\,nm})$ in dithionite-reduced particle suspensions (1 mg protein/mL) with bathophenanthrolinedisulfonate (50 μM) [30]. Reductive release of ferritin iron (25 μM total iron) during anaerobic incubation with SMP (0.1 mg protein/mL), NADH (0.2 mM) and DX (100 μM) was determined by continuously recording ($A_{540\,nm}-A_{575\,nm}$) using bathophenanthroline (50 μM) as a trap for ferrous iron in the presence of catalase (100 μg/mL) [19, 31]. The total amount of iron released from ferritin in 2 hr incubations was estimated after addition of ascorbic acid (100 μM) [32].

Covalent labeling of phospholipid in SMP with NHS-DX. A DX-derivative (NHS-DX) with an acylated sugar amino group and containing a reactive *N*-hydroxysuccinimide ester moiety was synthesized by reaction of DX (1 mg, 1.7 μmol) with the bifunctional cross-linker *bis*(sulfosuccinimidyl) suberate (5 mg, 8.7 μmol) in 0.5 mL of methanol/dimethylformamide (1:1, v/v) with 20 mM triethylamine [33, 34]. The derivative was purified by column chromatography on silica gel 60 eluted with chloroform/methanol (10:2, v/v). Yield was 50–60% on the basis of DX and purity was >90% by TLC on precoated silica gel 60 plates in the solvent system chloroform/methanol/water (32:12:2, v/v, R_F 0.38). SMP (5 mg of protein) in 10 mL of Buffer I was incubated for 1 hr at 30° with 100 nmol of NHS-DX followed by filtration through a Porapak Q column (1 × 5 cm, mesh 100–120, Waters, Millford, U.S.A.). Covalent binding of NHS-DX to the aminophospholipid PE in the particle membranes was 7–10 nmol/mg protein (~4% PE acylation) when determined by lipid extraction and TLC in the above solvent system (R_F 0.55 for the PE-DX derivative). Inhibition of cytochrome *c* oxidase activity during the labeling reaction was 10–20%. A detailed kinetic analysis of amino group acylation with NHS-DX in membrane lipid and protein is of relevance and will be presented in another publication.

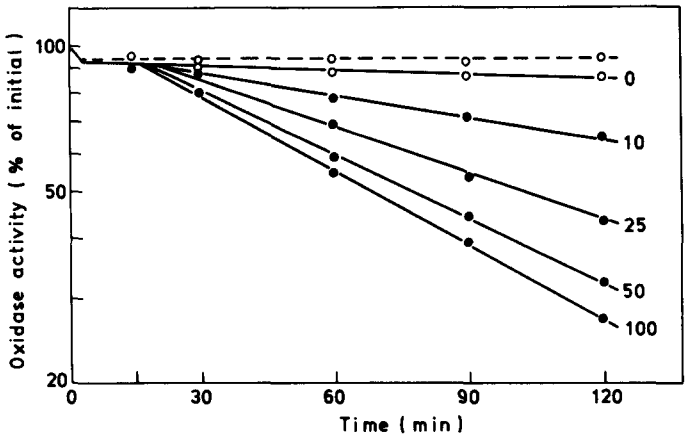

Fig. 1. Time-course of inactivation of cytochrome c oxidase activity. Submitochondrial particles were incubated in air-equilibrated buffer (0.1 mg protein/mL, 2.5 μM rotenone) and oxidase activity was determined at time intervals (spectrophotometric assay) after addition of 100 μM doxorubicin (O--O), 200 μM NADH (O——O) or 200 μM NADH plus 10–100 μM DX as indicated in the figure (●——●). Initial oxidase activity, 4544 ± 303 nmol/min/mg protein.

RESULTS

The effect of redox-cycling of DX on cytochrome c oxidase activity was examined in pig heart submitochondrial particles (SMP) incubated in air-equilibrated medium and supplemented with NADH as electron source for the respiratory chain. Rotenone-inhibited SMP were used in order to reduce consumption of NADH during the incubations. Redox-cycling of DX at 100 μM concentration increased the rate of NADH oxidation in the particles from 5.1 ± 0.8 to 8.7 ± 0.4 nmol/min/mg protein (means ± SD, $N = 7$), and superoxide radical formation was stimulated 10-fold from 0.9 ± 0.3 to 9.2 ± 1.2 nmol/min/mg protein ($N = 5$). A marked decrease in cytochrome c oxidase activity was measured during the course of DX redox-cycling. In 2 hr incubations with 100 μM DX, loss of activity was 70.6 ± 8.9% ($N = 8$) using the spectrophotometric assay for oxidation of exogenous ferrocytochrome c, and 63.1 ± 7.0% ($N = 5$) using the polarographic assay run with ascorbic acid and TMPD to determine oxidase activity with endogenous cytochrome c. The time-course of inactivation at various concentrations of DX (0–100 μM) is given in Fig. 1. Inactivation proceeded through an initial 30 min lag period corresponding to oxidation of about 15% of the total NADH (200 μM) in the reaction mixtures. The decrease in activity obtained with 100 μM DX alone was 7.2 ± 4.6% ($N = 8$), and occurred mainly during the first 5 min of incubation.

The content of chloroform/methanol extractable cardiolipin in the SMP was significantly reduced ($P < 0.02$) from 64.1 ± 3.5 to 51.0 ± 2.5 nmol/mg protein ($N = 4$) during 2 hr incubations with DX redox-cycling (100 μM DX), and DX-induced peroxidative breakdown of phospholipids was indicated by loss in polyunsaturated fatty acids in the total SMP-lipid (Table 1). Reduced minus oxidized difference spectra of the SMP showed no significant loss in heme a. Reactivation of oxidase activity was not obtained upon detergent solubilization of the SMP with Triton X-100/Tween 80 in the presence of exogenous cardiolipin in 100-fold molar excess to DX.

EDTA, DETAPAC, and the iron chelator desferrioxamine offered effective (75–95%) protection of oxidase activity and partial (30%) protection was obtained with the copper chelating diethyldithiocarbamic acid (Table 2). Control experiments confirmed that presence of the chelators was without major effect on the rate of DX redox-cycling as measured by superoxide radical formation (9.2 ± 0.3, 8.5 ± 0.9 and 6.8 ± 0.7 nmol/min/mg protein ($N = 4$) with 100 μM DX in the presence of EDTA (1 mM), DETAPAC (1 mM) or desferrioxamine (100 μM), respectively). Addition of 1 μM $FeCl_3$ to the incubation mixtures caused a drastic simulation in oxidase inactivation, whereas $CuCl_2$ and $MnCl_2$ were less effective. Addition of H_2O_2 up to 20 μM had no effect. Decomposition of H_2O_2 during the incubations due to contaminating catalase was <0.2 μM/min. Limited (30–45%) protection of activity was obtained by inclusion of catalase (Hydrogen peroxide: hydrogen peroxide oxidoreductase; EC 1.11.1.6) or scavengers of superoxide radicals: ferricytochrome c and superoxide dismutase (superoxide oxidoreductase; EC 1.15.1.1).

Treatment of the SMP suspensions with EDTA followed by filtration on Sepharose to remove contaminating ferritin and loosely bound iron from the particles prior to the incubations with DX redox-cycling, caused a 25–35% decrease in the rate of loss of oxidase activity. The amount of total SMP-iron not associated with heme or iron–sulfur proteins was found to be 0.5 ± 0.2 nmol/mg protein in four different particle preparations. This iron was not removed by EDTA treatment.

The dependence of cytochrome c oxidase inactivation by DX on oxygen concentration in the

Table 1. Analysis of major fatty acids in total phospholipid of incubated submitochondrial particles

	Fatty acids in total phospholipid (nmol/mg particle protein)				
	16:0	18:0	18:1	18:2	20:4
Control	228 ± 5	206 ± 5	202 ± 8	476 ± 13	203 ± 9
+ NADH	231 ± 12	204 ± 8	211 ± 10	483 ± 7	194 ± 5
+ NADH + DX	224 ± 10	197 ± 13	186 ± 9	439 ± 7	156 ± 3
+ NADH + DX + Ferritin	207 ± 14	133 ± 19	125 ± 18	211 ± 29	37 ± 4

Incubations were carried out for 2 hr in air-equilibrated Buffer I containing 0.1 mg SMP protein/mL and 2.5 μM rotenone (control), and with additions of NADH (200 μM), DX (100 μM), and ferritin (25 μM total iron). Values are means ± SD (N = 3).

Table 2. Effect of chelating agents, metal ions, and reactive oxygen scavengers on inactivation of cytochrome *c* oxidase activity in submitochondrial particles

Incubation system	Oxidase activity (% of initial)
Complete	36.2 ± 3.1
− doxorubicin	88.5 ± 0.3
+ EDTA (1 mM)	87.0 ± 2.5
+ DETAPAC (1 mM)	82.3 ± 2.8
+ desferrioxamine (1 μM)	67.0 ± 1.4
(10 μM)	74.6 ± 1.3
(100 μM)	71.3 ± 0.6
+ diethyldithiocarbamic acid (100 μM)	50.5 ± 2.7
+ Fe^{3+} (1 μM)	9.8 ± 0.7
+ Cu^{2+} (1 μM)	24.2 ± 1.6
+ Mn^{2+} (1 μM)	29.9 ± 3.1
+ H_2O_2 (20 μM)	35.1 ± 0.9
+ catalase (100 μg/mL)	52.5 ± 1.9
+ superoxide dismutase (20 μg/mL)	60.7 ± 1.9
(100 μg/mL)	57.3 ± 2.3
+ ferricytochrome *c* (50 μM)	54.3 ± 0.8

The complete incubation system (air-saturated Buffer I) contained 0.10 mg SMP protein/mL, 200 μM NADH, 2.5 μM rotenone and 100 μM doxorubicin. Oxidase activity was determined after 2 hr (spectrophotometric assay). Data are the means ± SD (N = 3–6). Initial oxidase activity, 4107 ± 288 nmol/min/mg protein.

particle suspensions is illustrated in Fig. 2. Protection of activity was obtained at <20 μM O_2 and was more than 95% effective after equilibration of the SMP with nitrogen. When the iron-containing protein ferritin (25 μM total iron) was included in the incubation mixtures, inactivation appeared to increase with decreased O_2 concentration to a maximum at <10 μM O_2 where also release of iron from the ferritin was maximal. Mobilization of up to about 10% of the total ferritin iron was obtained in the 2 hr incubations (data given in Fig. 2).

Rates of superoxide radical formation and loss of cytochrome *c* oxidase activity in the rotenone-inhibited SMP incubated with DX, *N*-acetylDX, or the nonanthracycline quinone menadione (2-methyl-1,4-naphthoquinone) are compared in Table 3. The latter two compounds are not charged and have a low binding affinity for anionic membrane sites such as cardiolipin. With menadione, a high rate of superoxide production was obtained with only limited effect on oxidase activity. This can be explained by an antioxidant property of menadione [35]. No significant effect of DX on superoxide production and NADH-dependent oxidase inactivation could be demonstrated after cross-linking to phosphatidylethanolamine in the particle membrane lipid. In a second set of experiments, incubations were carried out in which NADH oxidation in the SMP was inhibited by antimycin A instead of rotenone. Although superoxide radical formation in the presence of DX (50 μM) was higher than that obtained in the rotenone-inhibited SMP, inactivation of oxidase activity occurred at a significantly lower rate (Table 3).

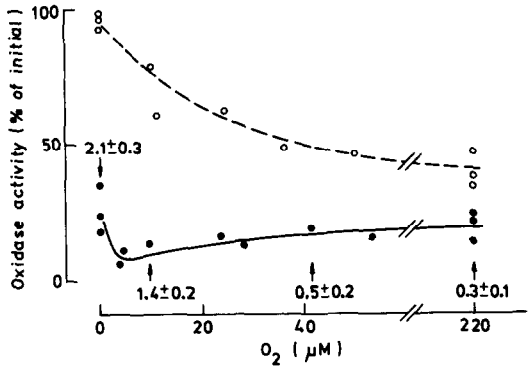

Fig. 2. Effect of O_2 concentration on inactivation of cytochrome *c* oxidase activity. Stirred suspensions of submitochondrial particles in buffer (0.1 mg protein/mL, 2.5 μM rotenone) equilibrated with air (220 μM O_2) or oxygen/nitrogen mixtures were incubated with doxorubicin (100 μM) plus NADH (200 μM) in the absence (O--O) and in the presence (●——●) of ferritin (25 μM total iron). Oxidase activity was determined after 2 hr (spectrophotometric assay). Results from determinations of iron release from ferritin are included in the figure (μM iron detected with bathophenanthroline/ascorbic acid after 2 hr incubation). Values are means ± SD (N = 3–4).

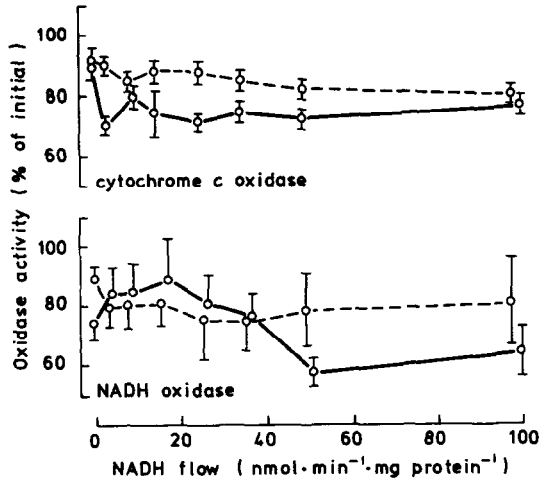

Fig. 3. Effect of NADH oxidation on inactivation of cytochrome *c* oxidase and NADH oxidase activity. Stirred suspensions of submitochondrial particles (0.1 mg protein/mL) in air-equilibrated buffer without respiratory chain inhibitors were incubated with continuous infusion of NADH at various rates in the absence (O--O) and in the presence (●——●) of doxorubicin (100 μM). Oxidase activities were assayed after 2 hr. Values are means ± SD ($N = 3$–6). Initial cytochrome *c* oxidase activity, 4477 ± 290 nmol/min/mg protein and initial NADH oxidase activity, 938 ± 166 nmol/min/mg protein.

We also investigated the effects of DX on the oxidase activity in the absence of respiratory chain inhibitors. With NADH oxidation controlled by continuous infusion of NADH at various rates into the stirred SMP suspensions, superoxide radical formation was <0.5 nmol/min/mg protein and neither cytochrome *c* oxidase nor NADH oxidase activity was significantly inhibited by 100 μM DX in 2 hr incubations (Fig. 3). The maximal infusion rate of 100 nmol NADH/min/mg protein (approx. 10% of the NADH oxidase activity) corresponded to addition of a total of 120 μM NADH in 2 hr. When NADH (0.2–20 mM) was included in the air-equilibrated SMP suspensions as a single addition at the start of 2 hr incubations, the results shown in Fig. 4 were obtained for the cytochrome *c* oxidase. Activity was significantly decreased ($P < 0.01$) by 100 μM DX at 20 mM NADH. However, this was accompanied by a marked inhibitory effect of NADH alone. The data in Fig. 4 also show that DX-dependent oxidase inactivation is not limited by NADH in the 0.2 to 20 mM range when rotenone is present to inhibit NADH oxidation through the respiratory chain.

Finally, in order to examine the role of reactive oxygen species in oxidase inactivation, we carried out incubations of the SMP in air-equilibrated buffer solutions with xanthine and various concentrations of xanthine oxidase (xanthine: oxygen oxidoreductase; EC 1.1.3.22) as a source of superoxide radicals and H_2O_2 [23]. A progressive inactivation occurred over a 2 hr period with an initial 30–60 min lag phase (Fig. 5). Radical generation rates of 0.8–1.6 μM/min, as measured by reduction of ferricytochrome *c*, gives a 60–75% loss in activity. This is comparable to the loss observed during redox cycling of 100 μM DX in the SMP which produce superoxide radicals at a rate of 1 μM/min (cf. Fig. 1). Oxidase activity was partially protected by inclusion of superoxide dismutase (25–35 % at 100 μg/mL) or catalase (50–60% at 100 μg/mL). Addition of urate up to 0.4 mM had no effect, confirming that inactivation relates to reduction of oxygen by the xanthine oxidase and not to an effect of the accumulating urate. The ratio of H_2O_2 to superoxide radical production in the xanthine oxidase/xanthine system was approx. 1.7:1. When H_2O_2 was added continuously at rates of 0.25–5 μM/min to incubations with SMP alone, it was found to have a direct inhibitory effect on the cytochrome *c* oxidase (Fig. 6).

DISCUSSION

The results presented here demonstrate that the potency of DX to impair the function of the mitochondrial cytochrome *c* oxidase is greatly enhanced during reduction and autoxidation of the drug in the 10–100 μM range. The finding that metal ion chelators offer effective protection to enzyme activity without affecting the rate of DX redox-cycling, shows that reduction of DX *per se* plays a minor role. Thus, impairment of oxidase function is not due to direct modifications of polypeptides in the enzyme complex or its lipid environment by DX in a reduced state. A role of DX-mediated oxygen reduction is supported by loss

Table 3. Comparison of superoxide production and cytochrome *c* oxidase inactivation in submitochondrial particles

Incubation system	Superoxide production (nmol/min/mg)	Oxidase activity (% of initial)
SMP + rotenone + NADH (200 μM)	0.9 ± 0.3	88.5 ± 0.3
+ DX (50 μM)	5.7 ± 0.3	31.0 ± 4.3
+ N-acetylDX (50 μM)	2.0 ± 0.2	67.2 ± 2.7
+ menadione (10 μM)	8.7 ± 0.5	87.8 ± 5.3
(100 μM)	46.6 ± 2.2	61.2 ± 1.7
+ DX-PE*	0.8 ± 0.2	85.0 ± 1.9
SMP + antimycin A + NADH (200 μM)	2.9 ± 0.7	90.9 ± 1.8
+ DX (50 μM)	11.6 ± 2.5	71.4 ± 6.0

The incubation system (air-saturated Buffer I) contained 0.10 mg SMP protein/mL and incubations were carried out for 2 hr in the absence or presence of respiratory chain inhibitors (rotenone, 2.5 μM; antimycin A, 0.4 μM). Superoxide production and oxidase activity (spectrophotometric assay) were determined as described in Materials and Methods. Values are the means ± SD (N = 3–7).

* DX covalently coupled to the particle phospholipid PE via a suberate linker arm (7.1 nmol DX/mg SMP protein).

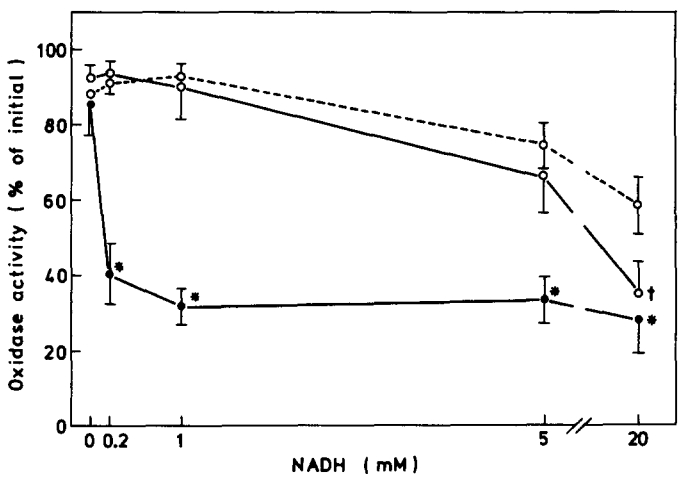

Fig. 4. Dependence of cytochrome *c* oxidase inactivation on NADH concentration. Incubation mixtures in air-equilibrated buffer contained submitochondrial particles (0.1 mg protein/mL) and NADH at the concentrations indicated. Reactions were carried out for 2 hr with no other additions (○--○), with 100 μM doxorubicin (○——○), and with 100 μM doxorubicin plus 2.5 μM rotenone (●——●). Values are the means ± SD (N = 4–5). Statistical difference from the control with NADH alone: *$P < 0.001$ (N = 5), †$P < 0.01$ (N = 5).

of activity also with oxygen-radicals generated in bulk medium by the xanthine oxidase reaction.

It is well-documented from previous investigations with microsomal and mitochondrial membrane preparations [36, 37], that aerobically reduced DX in combination with catalytic transition metal ions promotes a radical chain process leading to peroxidative decomposition of the membrane phospholipids. It is likely that initiation or propagation of this peroxidation reaction plays an important part in oxidase inactivation. Loss of oxidase activity is also irreversible with added cardiolipin and with detergent such as Tween 80 with ability to support maximal activity of the enzyme [26]. This result agrees with the findings by Hasinoff *et al.* [38], who measured irreversible loss in activity of detergent solubilized cytochrome *c* oxidase during incubation with a DX-iron complex. Inactivation of oxidase activities in SMP by iron complexes of DX does not depend on simultaneous electron transfer through the respiratory chain [27, 39], and possibly proceeds via self-reduction of the complexes in contact with the SMP membranes [39–42]. A role of endogenous iron in the present incubation system is indicated by protection of oxidase activity by the iron-binding siderophore desferrioxamine. Our results, however, do not allow us to determine whether this reactive iron, part of which appears to be associated with the SMP, interacts with DX. Minotti [43] used spectral

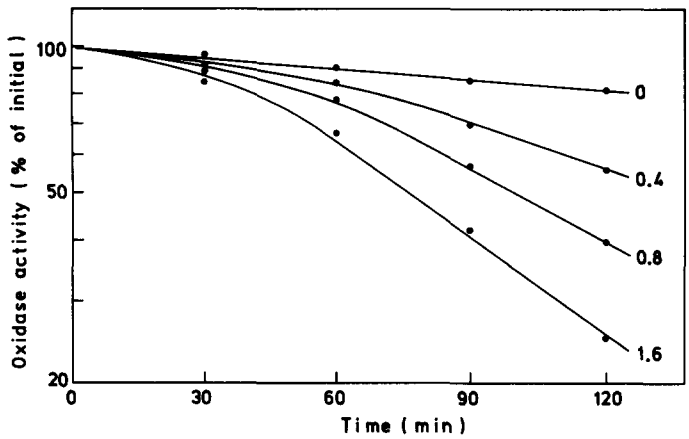

Fig. 5. Time-course of xanthine oxidase-dependent inactivation of cytochrome c oxidase activity. Incubation mixtures contained submitochondrial particles (0.1 mg protein/mL), xanthine (0.4 mM), and xanthine oxidase (0–0.005 units/mL) in air-equilibrated buffer. Radical generation rates (μM/min), as measured by ferricytochrome c reduction in parallel incubations without submitochondrial particles, are given in the figure.

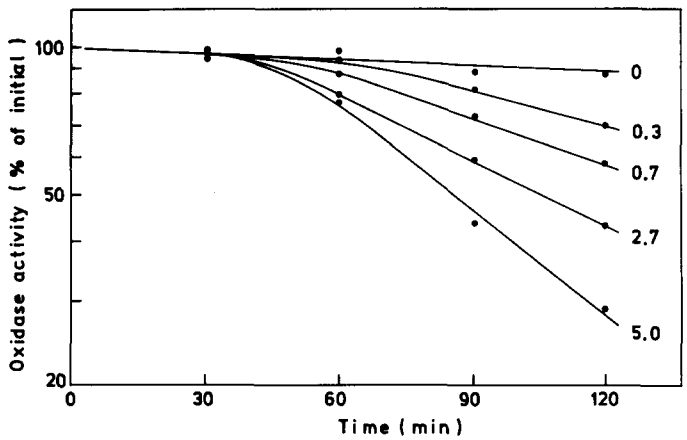

Fig. 6. Time-course of inactivation of cytochrome c oxidase activity by H_2O_2. Incubation mixtures in air-equilibrated buffer contained submitochondrial particles (0.1 mg protein/mL) and H_2O_2 was added by continuous infusion at the rates given in the figure (μM/min).

changes in the DX molecule [$\Delta(A_{660nm}-A_{530nm})$] to demonstrate direct reduction of iron in microsomal membranes by DX-semiquinone. Similar spectrophotometric measurements cannot be carried out with the SMP because of the low iron content and variable light scattering during incubation with DX.

The NADH-dependent oxidase inactivation is related in a complex manner to the rate of DX reduction determined as superoxide radical generation (Table 3). Inactivation is maximal with Complex I reduced in the presence of rotenone, and decreased in the presence of antimycin A and in incubations with an excess of NADH without a respiratory chain inhibitor. In a recent study by Marcillat *et al.* [44], cytochrome c oxidase activity of bovine heart SMP remained stable in 2 hr incubations with DX (50 μM) and excess NADH. Taken together these results support the idea that redox components of the reduced respiratory chain are able to protect the mitochondrial membrane against oxidative damage. An antioxidant, most likely reduced ubiquinone, is located between the rotenone- and antimycin-sensitive sites. Reduced ubiquinones act as direct free radical scavengers but may also serve to recycle tocopherol antioxidants in mitochondrial membranes [45, 46]. In this regard, it is important to note that ubiquinone reduction in mitochondria of heart muscle is in the 40 to 60% range [47]. No significant effect of DX on cytochrome c oxidase activity is observed with variation in the input rate of electrons from NADH to the uninhibited respiratory chain up to about 10% of the maximal NADH oxidase activity (Fig. 3), or in incubations with high (5 mM) NADH concentrations (Fig. 4).

This excludes the possibility that oxidase inactivation is related to turnover of the enzyme.

Goormaghtigh *et al.* [14, 48] reported on the function of the electrostatic DX-cardiolipin complex as an electron carrier between the mitochondrial Complex I, Complex III and cytochrome *c*. These results prompted us to analyse the redox behaviour of membrane bound DX. A kinetic analysis of inactivation data obtained after the initial 15–30 min lag period (Fig. 1) was carried out according to a simple reaction scheme [49]

$$DX + M \cdot Ox \underset{K_d}{\rightleftarrows} DX \cdot M \cdot Ox \xrightarrow[k_i]{} DX \cdot M \cdot Ox^*$$

in which the rapid formation of a reversible DX–membrane–oxidase complex ($DX \cdot M \cdot Ox$, dissociation constant K_d) is followed by an irreversible oxidase inactivation step ($Ox \rightarrow Ox^*$, rate constant k_i). Values for k_i were in the range 0.015–0.025/min and K_d 35–55 μM. Binding studies [2, 50], have indicated an apparent dissociation constant of 25–30 μM for the DX-cardiolipin complex in KCl–Hepes buffer. The calculated K_d values also compare to the reported K_m of 60 μM DX for DX induced superoxide radical formation in SMP [16]. To examine the possible reduction of lipid-bound DX in the SMP, the drug was covalently cross-linked via its sugar amino group to the membrane aminophospholipid PE up to about 10 nmol DX/mg protein, which is a relevant value for binding of DX to mitochondria *in vivo* [51]. As shown in Table 3, stimulation of superoxide radical production was not obtained with the lipid-bound DX derivative and NADH-dependent oxidase inactivation did not take place to any significant extent over a 2 hr period even under conditions with maximal reduction of Complex I in the presence of rotenone. We take this result to indicate a slow electron transfer from Complex I to the lipid-bound DX. Possible explanations are that introduction of the linker results in steric inhibition of the enzyme–drug interaction or that the drug binding site in Complex I is in equilibrium with the aqueous phase and not readily accessible through the membrane lipid. The latter conclusion is supported by previous results that no correlation is apparent between hydrophobicity and rate of reduction of a series of anthracycline derivatives [16, 22, 52, 53]. Model studies with cardiolipin-containing liposomes also indicate that only DX molecules with the anthraquinone moiety outside the lipid bilayer are able to be reduced by the isolated NADH:Q oxidoreductase (EC 1.6.99.3) [54].

The sarcoplasmic free-oxygen concentration in cardiac myocytes is in the 1–5 μM range as estimated from the reduction level of myoglobin [55]. At these low oxygen concentrations, iron in the intracellular iron storage protein ferritin is a better promoter of oxidase inactivation than is endogenous iron in the SMP preparations (Fig. 2). Vile and Winterbourn demonstrated that DX-dependent lipid peroxidation in microsomal membrane preparations with added ferritin is maximal at 5–10 μM oxygen, indicating an efficient reduction of ferritin-iron by the DX-semiquinone [56]. The initial rate of iron release from ferritin in our incubation system with SMP under anaerobic conditions is approximately 0.04 mol iron/mol NADH oxidized. Although this iron release is 6–12-fold lower than that obtained during reduction of DX with soluble NADPH-cytochrome P450 reductase (EC 1.6.2.4) [31, 57], it does indicate that reduced DX in the mitochondrial membrane is free to interact with the ferritin molecule. The bimolecular rate constant for reaction of the DX-semiquinone with molecular oxygen is $3.0 \times 10^8\ M^{-1}\ sec^{-1}$ [58], which gives a half-life of 2.3 msec for DX-semiquinone in the aqueous phase at 1 μM oxygen. The mean displacement of the semiquinone in this time interval as calculated from the Einstein–Smoluchowski equation, is about 2 μm. The direct interaction of ferritin with binding sites in the mitochondrial membranes [59] is thus unlikely to be rate limiting for iron release by the DX-semiquinone at intracellular oxygen concentrations.

In summary, this study provides detailed information about the role of DX and iron in inactivation of the cytochrome *c* oxidase in isolated mitochondrial membranes. It is evident that the binding of DX to membrane lipids and the reduction–oxidation cycle of the drug itself has little direct damaging effect on the enzyme or its essential phospholipid (cardiolipin) environment. The results strongly suggest that irreversible oxidase inactivation occurs in conjugation with the formation of oxygen free radicals and an iron-dependent peroxidation reaction. The implications of this result to the *in vivo* situation is, however, complicated by the fact that although the affinity of DX for iron is sufficient for transfer of ferritin- and transferrin-iron to DX [32, 60], the interactions of the drug with intracellular iron has not been clarified. Also, DX may react differently with the reduced respiratory chain in rotenone-inhibited SMP and in intact mitochondria with State 4 respiration. There is a possibility that fragmentation of the mitochondrial membrane by sonication leads to alterations in the ability of respiratory chain components to transfer electrons to DX [61]. Further, it must be noted that a slow rate of oxidase inactivation in the SMP-system not necessarily indicates that this slow process is without importance *in vivo*. Previous studies have demonstrated extensive loss of cytochrome *c* oxidase activity of heart mitochondria during the course of high-dose DX toxicity in mice [62, 63]. How this oxidase inactivation relates to iron dependent free radical reactions, activation of proteolytic enzymes functioning in the mitochondria [64] and decreased protein synthesis, remains to be elucidated.

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