Secondary Alcohol Metabolites Mediate Iron Delocalization in Cytosolic Fractions of Myocardial Biopsies Exposed to Anticancer Anthracyclines

Novel Linkage Between Anthracycline Metabolism and Iron-induced Cardiotoxicity

Giorgio Minotti,* Anna Franca Cavaliere,‡ Alvaro Mordente,§ Marco Rossi,‖ Rocco Schiavello,‖ Roberto Zamparelli,‖ and GianFederico Possati¶

*Departments of *Pharmacology, ‡Obstetrics and Gynecology, §Biological Chemistry, ‖Anesthesiology and Intensive Care, and ¶Cardiac Surgery, Catholic University School of Medicine, Largo F. Vito 1, 00168 Rome, Italy*

Abstract

The cardiotoxicity of doxorubicin (DOX) and other quinone-containing antitumor anthracyclines has been tentatively attributed to the formation of drug semiquinones which generate superoxide anion and reduce ferritin-bound Fe(III), favoring the release of Fe(II) and its subsequent involvement in free radical reactions. In the present study NADPH- and DOX-supplemented cytosolic fractions from human myocardial biopsies are shown to support a two-step reaction favoring an alternative mechanism of Fe(II) mobilization. The first step is an enzymatic two-electron reduction of the C-13 carbonyl group in the side chain of DOX, yielding a secondary alcohol metabolite which is called doxorubicinol (3.9±0.4 nmoles/mg protein per 4 h, mean±SEM). The second step is a nonenzymatic and superoxide anion-independent redox coupling of a large fraction of doxorubicinol (3.2±0.4 nmol/mg protein per 4 h) with Fe(III)-binding proteins distinct from ferritin, regenerating stoichiometric amounts of DOX, and mobilizing a twofold excess of Fe(II) ions (6.1±0.7 nmol/mg protein per 4 h). The formation of secondary alcohol metabolites decreases significantly ($P < 0.01$) when DOX is replaced by less cardiotoxic anthracyclines such as daunorubicin, 4′-epi DOX, and 4-demethoxy daunorubicin (2.1±0.1, 1.2±0.2, and 0.6±0.2 nmol/mg protein per 4 h, respectively). Therefore, daunorubicin, 4′-epi DOX, and 4-demethoxy daunorubicin are significantly ($P < 0.01$) less effective than DOX in mobilizing Fe(II) (3.5±0.1, 1.8±0.2, and 0.9±0.3 nmol/mg protein per 4 h, respectively). These results highlight the formation of secondary alcohol metabolites and the availability of nonferritin sources of Fe(III) as novel and critical determinants of Fe(II) delocalization and cardiac damage by structurally distinct anthracyclines, thus providing alternative routes to the design of cardioprotectants for anthracycline-treated patients. (*J. Clin. Invest.* 1995. 95:1595–1605.) Key words: Doxorubicin • anthracyclines • iron • free radicals • cardiotoxicity

Introduction

The formation of an intracellular pool of free iron can be viewed as a sort of twin-edged sword. Some iron is necessary for cell functions, however, excess iron catalyzes free radical reactions that degrade polyunsaturated fatty acids, proteins, and nucleic acids (1, 2). This dual function of iron is particularly evident with Fe(II), which is more active than Fe(III) in generating hydroxyl radicals, ferryl ions, or equally damaging iron–oxygen complexes (1). Under physiologic conditions excess Fe(II) is sequestered by ferritin, a shell-shaped multisubunit protein which is placed in the cytosolic milieu and incorporates Fe(III) by oxidizing Fe(II) with oxygen (3). Ferritin would subsequently release iron only when electron donors accumulate and reduce Fe(III) to Fe(II) (4, 5). These premises imply that free radical reactions and tissue damage must be preceded by disturbances of ferritin iron movements, resulting in a pathologic process of Fe(II) delocalization. One such process appears to mediate the cardiotoxicity of doxorubicin (DOX),[1] an anthracycline antibiotic which is active against several tumors but also causes a potentially fatal cardiomyopathy when the cumulative dose exceeds 550 mg/m^2 (6). Clinical studies indicate that the incidence and severity of this cardiotoxicity are significantly lowered by the concurrent administration of dexrazoxane (6, 7), a bispiperazinedione which hydrolyzes intracellularly and liberates a diacid diamide that chelates iron (8). The cardioprotective effect of dexrazoxane is nevertheless incomplete (9), perhaps because a complex of iron with the diacid diamide still generates some fluxes of hydroxyl radicals (10).

Doxorubicin is comprised of a quinone-containing tetracycline ring and a two-carbon side chain, having a carbonyl moiety at C-13 and a primary alcohol at C-14; an aminosugar (daunosamine) is attached by glycosidic bond to the C-7 of the tetracycline ring (Fig. 1). In vitro studies have shown that NAD(P)H oxidoreductases of mitochondrial, nuclear, and microsomal membranes support a one electron redox cycling of DOX that may be relevant to the processes of iron delocalization and cardiac damage. In fact, one electron addition to the quinone group forms an unstable semiquinone free radical that regenerates DOX by reducing and releasing iron stored in the ferritin core (11, 12). Under aerobic conditions, the release of Fe(II) is preceded by a redox coupling of DOX semiquinone free radical with oxygen, yielding a superoxide anion ($O_2^{\cdot -}$) as the ultimate reductant for ferritin-bound Fe(III) (11, 12). Notewor-

Address correspondence to Dr. Giorgio Minotti, Department of Pharmacology, Catholic University School of Medicine, Largo F. Vito 1, 00168 Rome, Italy. Phone: 39-6-301-54367; FAX: 39-6-305-7968.

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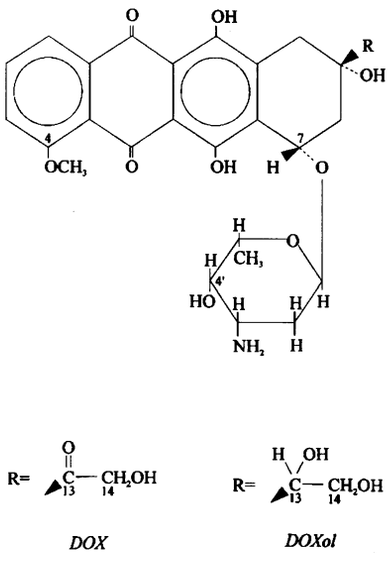
1. *Abbreviations used in this paper:* CPB, cardiopulmonary bypass; DNR, daunorubicin; DNRol, daunorubicinol; DOX, doxorubicin; DOXol, doxorubicinol; H, heavy; L, light; $O_2^{\cdot -}$, superoxide anion; rHF, recombinant heart-type ferritin; rLF, recombinant liver-type ferritin.



DOX DOXol

Figure 1. Structures of DOX and DOXol.

thy, cardiomyocytes have relatively little SOD activity as compared with other cell types, mostly because of a selective decrease of the copper-requiring cytosolic enzyme (13). The deficiency in SOD might explain how cardiomyocytes become the targets of a drug which releases Fe(II) by forming $O_2^{\cdot-}$.

In vitro and in vivo studies (14) have also shown that the side chain C-13 carbonyl group of DOX is liable to a two electron reduction, yielding a secondary alcohol which is called doxorubicinol (DOXol) (see also Fig. 1). This reaction is catalyzed by pancytosolic enzymes that utilize NADPH as a source of reducing equivalents and have been classified as aldo–keto reductases [EC 1.1.1.21] (15) or carbonyl reductases [EC 1.1.1.184] (16). Doxorubicinol is the primary circulating metabolite in DOX-treated patients (17) and laboratory animals (18). Perhaps more importantly, the cardiac performance of DOX-treated animals usually decreases at a time when DOXol has reached its maximum intramyocardial concentration (19, 20), suggesting that this metabolite might be intimately involved in the molecular mechanisms of cardiac damage. Pharmacokinetic studies in DOX-treated animals indicate that cardiac accumulation of DOXol reflects intramyocardial drug metabolism rather than uptake of the metabolite from the blood stream (14, 20, 21).

Ethical and practical reasons preclude fine needle biopsies of sufficient size to evaluate DOXol formation and mechanism(s) of toxicity in the heart of DOX-treated patients. Therefore, we have studied the cytosolic metabolism of DOX and other anthracyclines in myocardial samples from patients undergoing open-chest cardiac surgery or obtained at autopsy. The aims of these studies were to demonstrate that: (*a*) human cardiomyocytes can enzymatically reduce DOX to DOXol; (*b*) this metabolite contributes to cardiac damage by reductively delocalizing iron; and (*c*) differences in secondary alcohol formation and iron mobilization can underlie clinical differences between DOX and less cardiotoxic anthracyclines. The results indicate that the cardiotoxicity of DOX and other anthracyclines strictly reflects intramyocardial formation of secondary alcohol metabolites which reductively delocalize iron from Fe(III)-binding proteins distinct from ferritin. These findings provide new clues to the pharmacologic cardioprotection of anthracycline-treated patients.

Methods

Materials. Lactose-free anthracyclines were kindly provided by Dr. Antonio Suarato (Pharmacia-Farmitalia Carlo Erbas, S.p.A. Milan, Italy). Ammonium sulfate (ultrapure grade, $Fe^{III} < 0.5$ ppm) was purchased from Schwarz/Mann (Cleveland, OH). EDTA was from Merck & Co., Inc. (Darmstadt, Germany), whereas $FeCl_2$ and $NaBH_4$ were from Fluka AG (Buchs, Switzerland). Chelex 100 was from Bio-Rad Laboratories (Richmond, CA). Sephadex G-25 and electrophoresis reagents were purchased from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden), whereas Sepharose 6B, human holotransferrin, bovine erythrocyte CuZn SOD (EC 1.15.1.1), and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Unless otherwise indicated, all the experiments were carried out in 0.3 M NaCl, carefully adjusted to pH 7.0 just before use. This was done to avoid ligand-catalyzed interactions of most common buffers with iron (1, 22). Although unbuffered, the pH of the reaction mixtures did not vary throughout the experimental time. All the solutions were prepared with double distilled water which had been passed through a Synbron Barnstead NANOpure II system (Idrotecnica S.p.A., Bolzaneto, Italy). Subsequent ion exchange chromatography on a column of Chelex 100 was performed to remove trace metals.

Patients and myocardial samples. Small atrial samples (~ 0.13 g) were taken from male or female patients undergoing aorto-coronary bypass grafting. Briefly, bioptic fragments of normothermic beating myocardium were taken from the lateral aspect of excluded right atrium before tying the purse string of atrial cannulation. These samples were collected 15–20 min before or 5–10 min after cardiopulmonary bypass (CPB). During CPB, myocardial preservation was obtained by infusion of a St. Thomas Hospital–modified cardioplegic solution (23). The median age of patients was 58±8 yr (n = 15) or 59±7 yr (n = 25) for the pre- or post-CPB samples, respectively. The biopsies were stored at −80°C until they formed two pools of 2.7 or 4.4 g for the pre- or post-CPB samples, respectively. Larger samples (~3 g) were obtained by recovering right auricolas from a 66-yr-old male and a 64-yr-old female patient undergoing Maze's correction of drug-unresponsive atrial fibrillation (24). Informed consent was obtained from all patients. Ventricular postmortem samples (5–10 g) were provided by authorized pathologists during the autopsy of male or female individuals with morphologically normal myocardium and no clinical history of: (*a*) acute myocardial infarction, severe cardiosclerosis, or other cardiomyopathies; (*b*) inherited or acquired hemochromatosis; and (*c*) highly contagious infectious diseases. Selected samples were obtained from patients (n = 20, median age = 53±3 yr) died of: (*a*) sudden rupture of abdominal or intracranial aneurysms (n = 9); (*b*) unresectable or relapsed brain tumors (n = 5); and (*c*) biliary or pancreatic cancer (n = 6). All samples were collected 24 h after death. Anthracycline-treated patients were routinely excluded from the study to avoid sample perturbation by changes in drug-metabolizing enzymes or anomalous modifications of iron content and distribution.

Preparation of cytosols. The two pools of ex vivo pre- or post-CPB samples and individual 3–5 g samples from postmortem donors or Maze's patients were thawed in 0.3 M NaCl, pH 7.0, 4°C, minced with scissors, and disrupted first by four 15-s bursts in a blade homogenizer (UltraTurrax; Kunkle & Janke GmbH & CokG, Staufen, Germany) and then by four strokes in a homogenizer (Potter-Elvehjem; Thomas Scientific, Swedesboro, NJ) using a loose teflon pestle. Tissue homogenates were centrifuged for 30 min at 16,000 *g* and the supernatants were further centrifuged for 30 min at 25,000 *g*. The resulting supernatants were eventually ultracentrifuged for 90 min at 105,000 *g*. Dialyzed 105,000 *g* supernatants were unstable on freezing and thawing and formed gross particulates upon incubation with NADPH and/or DOX, precluding spectrophotometric assays for Fe(II) mobilization. Therefore, 105,000 *g* supernatants were routinely stirred overnight with 65% ammonium sulfate. After 30 min centrifugation at 10,000 *g*, the precipitates were dissolved in 1–2 ml of 0.01 M phosphate buffer, pH 7.4, and loaded onto a (0.8 × 10 cm) calcium hydroxyapatite column previously equilibrated with the same buffer. This procedure was chosen because

it is known to increase the anthracycline aldo–keto reductase activity of rat liver cytosol (15). The column was washed with a threefold excess (vol/vol) of the equilibration buffer and the effluents were stirred overnight with 65% ammonium sulfate. After 30 min centrifugation at 10,000 *g*, the precipitates were dissolved in 3–4 ml of double-distilled water and dialyzed first against two 1-liter changes of 0.3 M NaCl-1 mM EDTA (to remove adventitious iron), and then against two 1-liter changes of 0.3 M NaCl (to remove EDTA and EDTA-iron complexes). These "unmodified cytosols" could be stored at −20°C and thawed without appreciable formation of gross particulates. Likewise, these cytosols were stable upon reconstitution with NADPH and/or DOX. All preparations were essentially free of mitochondrial cytochrome *c* oxidase activity, and microsomal or nuclear NADPH-cytochrome *c* or P450 reductase activities (10, 12, 25). In selected experiments, unmodified cytosols were subsequently treated with thioglycolic acid (which reduces Fe^{III} to Fe^{II}) and bathophenanthroline (which forms a stable complex with Fe^{II}). Briefly, the incubations (2–5 ml final vol) contained unmodified cytosols (3 mg protein/ml), bathophenanthroline disulfonate (0.5 mM), and thioglycolic acid (0.5%, vol:vol) in 0.3 M NaCl, pH 7.0. After 1 h stirring on ice, the mixtures were loaded onto a (2.5 × 16 cm) Sephadex G-25 column to separate cytosolic proteins from thioglycolic acid and the bathophenanthroline-Fe(II) complex. Both column equilibration and sample elution were performed with 0.3 M NaCl, pH 7.0. 5-ml fractions were collected at the flow rate of 5 ml/min and monitored for proteins by measuring absorbance at 280 nm. Protein-containing fractions were pooled, concentrated by ultrafiltration in 10-kD exclusion limit Minicon cells (Amicon Corp., Danvers, MA), and shown not to contain bathophenanthroline-chelatable Fe(II) upon reincubation with thioglycolic acid. Therefore, these cytosols will be referred to as "iron-depleted." In other experiments, "iron-reconstituted" cytosols were prepared by taking advantage of the ability of iron-depleted cytosols to incorporate Fe(III) by oxidizing Fe(II). The incubations (2–5 ml final vol) contained iron-depleted cytosols (3 mg protein/ml) and $FeCl_2$ (0.5 mM) in 0.3 M NaCl, pH 7.0, 37°C. The reactions were terminated at regular times by the sequential addition of bathophenanthroline disulfonate (2.5 mM) and EDTA (1 mM), to chelate unreacted Fe(II) and remove loosely bound Fe(III), respectively. After 1 h stirring on ice, the mixtures were chromatographed on Sephadex G-25 to separate cytosolic proteins from bathophenanthroline-Fe(II) and EDTA-Fe(III) complexes. The iron-reconstituted cytosols with the highest Fe(III) content will be referred to as "iron-loaded."

Assays for anthracycline metabolites. Anthracycline metabolism was studied in incubations (0.5–1 ml final vol) containing cytosols (3 mg protein/ml), drugs (1 mM) and NADPH (1 mM) in 0.3 M NaCl, pH 7.0, 37°C. After a 4-h incubation in a metabolic shaker (Dubnoff; Vismara Associate S.p.A., Milan, Italy), the reactions were terminated by the addition of a fourfold excess of $CH_3OH/CHCl_3$ (1:1). After low-speed centrifugation, polar and apolar metabolites were recovered from CH_3OH or $CHCl_3$ phases, lyophilized, and resuspended in a minimum volume of the appropriate solvent. These samples were analyzed by two-dimensional TLC on (0.25/0.5 mm) Silica Gel Plates 60 F-254 (Merck & Co., Inc., Darmstadt, Germany). Unmodified anthracyclines and polar C-13 secondary alcohol metabolites were separated using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (80:20:14:6) in either dimension (26). Apolar aglycones were separated using $CHCl_3/CH_3OH/CH_3COOH$ (100:2:5) in the first dimension and $CH_3COOC_2H_5/CH_3CH_2OH/CH_3COOH/H_2O$ (80:10:5:5) in the second dimension (26). Fluorescent spots corresponding to the polar or apolar metabolites were eluted with 100% CH_3OH or $CHCl_3$, respectively, and assayed fluorimetrically against standards of unmetabolized anthracyclines in a luminescence spectrometer (LS 5; Perkin-Elmer Corp., Norwalk, CT), as described in detail by Takanashi and Bachur (26).

Enzymatically generated DOXol (referred to as "enzymatic" DOXol) was identified by cochromatography with authentic DOXol from *Streptomyces peucetius* var. *caesius*, or DOXol prepared by $NaBH_4$ reduction of DOX, referred to as "chemical" DOXol. The latter was prepared by established procedures (26) and purified by two-dimensional TLC for polar metabolites. Enzymatic (4-demethoxy)daunorubicinol (DNRol) and 4′-epi DOXol were identified by cochromatography with chemical analogs prepared by $NaBH_4$ reduction of the parent compounds. Doxorubicin(ol) aglycones were identified vs standards prepared by thermoacid hydrolysis of DOX and chemical DOXol (26) and purified by two-dimensional TLC for apolar metabolites. Doxorubicin hydroquinone standards (the products of a two-electron reduction of the quinone moiety) were prepared enzymatically by incubating DOX with NADPH and spinach leaf $NADP^+$-ferredoxin oxidoreductase (EC 1.18.1.2) (27). Only 7-deoxy DOX hydroquinone aglycone was formed, in keeping with previous reports that a two electron reduction of the quinone moiety is accompanied by heterolysis of the glycosidic bond between daunosamine and the tetracycline ring (11, 27). Purification of 7-deoxy DOX hydroquinone aglycone was also obtained by two-dimensional TLC for apolar aglycones, where it migrated with R_f of 0.01 and 0.24 in the first or second dimension, respectively. These values were lower than those determined for any DOX(ol) oxy- or deoxy-aglycone (26). 7-deoxy DOX aglycone and 4-demethoxy DOXol aglycone were identified by ≥ 95% agreement of the R_f determined by us with those determined by others under comparable conditions (26).

Assays for Fe(II) mobilization. Ferrous iron mobilization was studied in incubations (1 ml final vol) containing cytosols (1 mg protein), anthracyclines (0.25 mM), and bathophenanthroline disulfonate (0.5 mM) in 0.3 M NaCl, pH 7.0, 37°C. The reactions were started by adding NADPH (0.25 mM) and the formation of a bathophenanthroline-Fe(II) complex was monitored spectrophotometrically at 534 nm vs a reference cuvette containing all the reactants but the chelator (28). Standard curves with known amounts of bathophenanthroline-chelated $FeCl_2$ were used for calculations, and control experiments showed that the bathophenanthroline-Fe(II) complex produced from cytosols was spectrally identical to authentic complexes. With the exception of the data in Fig. 2, all values were corrected for iron mobilization by NADPH or anthracyclines added individually. Where indicated, iron mobilization was monitored under nonenzymatic conditions by replacing NADPH and anthracyclines with purified C-13 metabolites. The latter had been extensively washed with methanol, lyophilized, and eventually suspended in 0.3 M NaCl, pH 7.0, just before use.

Ferritin preparation. Heart ferritin is composed primarily of heavy (H) subunits whereas liver and spleen ferritins are composed primarily of light (L) subunits (3). Recombinant human H or L ferritin homopolymers were kindly provided by Dr. Paolo Arosio (Biotechnology Department, Istituto Scientifico San Raffaele, Milan, Italy). Ferritin expression and purification were as described by Levi et al. (3). Native H or L samples contained 65 or 9 atoms Fe(III)/molecule, respectively. Therefore, apoferritins were prepared and incubated with iron to obtain ferritins with higher Fe(III): protein ratios. Apoferritins were obtained by treating ferritins with thioglycolic acid and bathophenanthroline, as described for the preparation of iron-depleted cytosols. Ferric iron incorporation was obtained by taking advantage of the ferroxidase activity of apoferritin or ceruloplasmin. Briefly, apoferritins (0.3 μM) and $FeCl_2$ (0.5 mM), plus or minus ceruloplasmin (0.3 μM), were incubated for 1 h in 0.3 M NaCl, pH 7.0, 37°C. The reactions were terminated by the sequential addition of bathophenanthroline and EDTA, as described for the preparation of iron-reconstituted cytosols. In these experiments, thioglycolic acid, bathophenanthroline-Fe(II), and EDTA-Fe(III) were removed by extensive dialysis of the (apo)ferritin samples against 0.3 M NaCl, pH 7.0. The H or L ferritins reconstituted via their own ferroxidase activity had iron:protein ratios of 95 or 32, respectively, whereas H and L ferritins reconstituted via the ferroxidase activity of ceruloplasmin had iron: protein ratios of 110 or 260, respectively. Ceruloplasmin was purified from rat blood plasma according to Ryan et al. (29). Ferritins were separated from ceruloplasmin by gel filtration on a (1.8 × 27 cm) Sepharose 6B column equilibrated with 0.3 M NaCl, pH 7.0, and eluted at the flow rate of 0.74 ml/min.

Affinity and gel filtration chromatographies of cytosolic iron and recombinant ferritins. 8 mg protein of unmodified or iron-loaded cytosols from ex vivo Maze's samples (corresponding to 64 or 184 nmol Fe^{III}, respectively) were dialyzed overnight against two 1-liter changes of 0.15 M NaCl-10 mM Tris HCl, pH 7.4, concentrated to 1 ml by

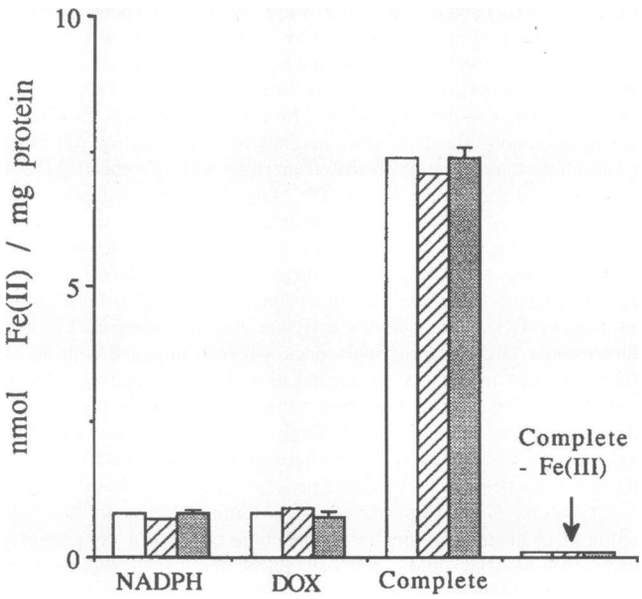


Figure 2. NADPH- and DOX-dependent Fe(II) mobilization in human heart cytosols. Unmodified or iron-depleted cytosols from ex vivo or postmortem samples were assayed for NADPH- and/or DOX-dependent Fe(II) mobilization as described in Methods. Values are those determined at 4 h and are taken from representative experiments or four separate determinations (mean±1 SE). -Fe(III), iron-depleted cytosols; ex vivo □ pre CPB; ■ post CPB; ■ post mortem.

ultrafiltration, and loaded onto a (1.3 × 2.5 cm) CNBr-Sepharose 4B column which had been equilibrated with the same buffer. The column had been conjugated with 1 mg of antirecombinant heart-type ferritin (rHF) monoclonal RHO2 IgG_1 (30) and 1 mg of antirecombinant liver-type ferritin (rLF) monoclonal L03 IgG_{2b} (31). Samples were eluted with 9 ml of the equilibration buffer and the effluent was rechromatographed twice. The final effluent (referred to as "Tris effluent") was concentrated to 1 ml by ultrafiltration and chromatographed on Sepharose 6B as already described for the separation of ferritins from ceruloplasmin. Fractions (0.9 ml) were collected and monitored for absorbance at 280 nm. Protein-containing fractions were subsequently assayed for nonheme iron by reconstituting 0.3-ml aliquots with thioglycolic acid (0.5% vol:vol), bathophenanthroline disulfonate (0.5 mM), and 0.3 M NaCl in a final volume of 0.9 ml. After vigorous mixing, the iron content was determined by measuring the absorbance of the bathophenanthroline-Fe(II) complex at 534 nm. In other experiments, the antiferritin CNBr-Sepharose 4B column was charged with a mixture of self-reconstituted rHF (174 μg protein −30 nmol iron) and ceruloplasmin-reconstituted rLF (166 μg protein −90 nmol of iron). The column was washed with 9 ml equilibration buffer and ferritins were eluted with 4.5 M $MgCl_2$ (32) by monitoring the effluent for absorbance at 280 nm. Fractions with absorbance ("Mg effluent") were pooled, dialyzed extensively against 0.3 M NaCl, and concentrated to 1 ml by ultrafiltration in Centripep 30 (Amicon Corp., Danvers, MA). These samples were eventually chromatographed on Sepharose 6B and analyzed for iron as described for the Tris effluent of cytosols.

Other assays. Spontaneous or cytosol-induced Fe(II) oxidation was studied in incubations (1 ml final vol) containing $FeCl_2$ (0.5 mM) ±iron-depleted cytosols (3 mg protein/ml) in 0.3 M NaCl, pH 7.0, 37°C. Aliquots (0.1 ml) were taken at regular times and reconstituted with bathophenanthroline disulfonate (0.5 mM) and 0.3 M NaCl, in a final volume of 1 ml. After mixing, these samples were assayed for absorbance of the bathophenanthroline-Fe(II) complex at 534 nm. The ferric iron content of all cytosol and ferritin preparations was routinely measured using thioglycolic acid as the reductant for Fe(III) and bathophenanthroline as the chromophore for Fe(II). Omission of thioglycolic acid always prevented the formation of the bathophenanthroline-Fe(II) complex, indicating that all the assayable iron was in a ferric form. Unmodified cytosols from postmortem samples contained 8.4±0.33 nmol Fe(III)/mg protein (n = 8). The two preparations from ex vivo Maze's samples contained 8 and 9.2 nmol Fe(III)/mg protein, whereas representative preparations from pools of pre- or post-CPB samples contained 7.8 or 8.4 nmol Fe(III)/mg protein, respectively. Proteins were measured by the bicinchoninic acid method (33). SDS-PAGE analysis under reducing conditions was performed according to Laemmli (34) using 15% polyacrylamide gels. Unless otherwise indicated, all data are expressed as the arithmetic mean±SEM. Statistical analyses were performed by paired Student's t test. Differences were considered significant when $P < 0.05$. All other conditions are indicated in the figure legends.

*Table I. Doxorubicinol Formation in NADPH-supplemented Unmodified or Iron-depleted Cytosols from Human Myocardial Samples**

Sample	Unmodified cytosol	Iron-depleted cytosol
	nmol DOXol/mg protein per 4h	
Postmortem	0.7±0.2	3.9±0.4‡
Ex vivo		
pre-CPB	0.7	3.9
post-CPB	0.8	3.7

* NADPH- and DOX-supplemented unmodified or iron-depleted cytosols from ex vivo or postmortem samples were assayed for DOXol as described in Methods. Values are those determined at 4 h and are means±SE (n = 4). Values with ex vivo samples are taken from representative experiments. ‡ Significantly different from unmodified cytosol ($P < 0.01$).

Results

(a) NADPH- and DOX-dependent Fe(II) mobilization in unmodified or iron-depleted cytosols. NADPH and DOX-supplemented cytosolic fractions from human myocardium were found to release Fe(II). As shown in Fig. 2, comparable results were obtained with cytosols from selected postmortem samples and cytosols from ex vivo pre- or post-CPB samples. NADPH or DOX per se mobilized very little Fe(II); moreover, the mobilization of Fe(II) was not observed when the cytosols had previously been depleted of nonheme Fe(III) (see also Fig. 2). These results showed that the mobilization of Fe(II) was mediated by DOX metabolites which reduced nonheme Fe(III).

(b) NADPH-dependent DOX metabolism in unmodified or iron-depleted cytosols. NADPH-supplemented unmodified cytosols from postmortem or ex vivo samples were found to reduce DOX to DOXol, however, the yield of DOXol increased dramatically when the cytosols had previously been depleted of nonheme Fe(III) (Table I). Omission of NADPH or cytosols abolished DOXol formation, indicating that DOX reduction was mediated by an enzymatic mechanism. Doxorubicin-metabolizing enzymes are also known to cleave the glycosidic bond between daunosamine and the tetracycline ring (26), therefore, DOX- and DOXol aglycones were measured in unmodified or iron-depleted cytosols. As shown in Table II, NADPH-supplemented unmodified cytosols from postmortem samples were capable of forming DOX aglycone and 7-deoxy DOX aglycone,

Table II. NADPH-dependent DOX Metabolism in Unmodified or Iron-depleted Cytosols: Comparison of DOXol with Aglycone Metabolites

Metabolite	Unmodified cytosol	Iron-depleted cytosol
	nmol/mg protein per 4 h	
DOXol	0.7±0.2	3.9±0.4*
DOX-ag	0.8±0.2	0.8±0.1‡
7-deoxy DOX-ag	0.4±0.3	0.5±0.4‡
4-demethoxy DOXol-ag	0.3±0.1	n.d.
DOXol-ag	n.d.	n.d.
7-deoxy DOX hydroquinone-ag	n.d.	n.d.

NADPH- and DOX-supplemented unmodified or iron-depleted cytosols from postmortem samples were assayed for DOXol and apolar aglycones as described in Methods. Values are those determined at 4 h and are means±SE (n = 4). * Significantly different from unmodified cytosol ($P < 0.01$). ‡ NS vs unmodified cytosol; ag, aglycone; n.d., not detectable.

however, neither metabolite increased significantly upon iron removal. 4-demethoxy DOXol aglycone was formed by unmodified but not by iron-depleted cytosols, whereas DOXol aglycone and 7-deoxy DOX hydroquinone aglycone were not formed by either cytosol preparation (see also Table II). Hence, DOXol was the only DOX metabolite which increased upon iron removal. Comparable results were obtained with cytosols from ex vivo pre- or post-CPB samples (not shown).

(c) Enzymatic and non-enzymatic DOXol-dependent mobilization of Fe(II) in unmodified or iron-reconstituted cytosols. Figure 3 *A* shows that 90% of Fe(II) could be recovered with bathophenanthroline after a 3-h incubation in unbuffered saline under an air atmosphere. The addition of iron-depleted cytosols decreased the recovery of bathophenanthroline-chelatable Fe(II) and the net loss of Fe(II) was paralleled by a tenacious binding of Fe(III) to cytosolic proteins (Fig. 3 *B*). These results showed that cytosols could incorporate Fe(III) by oxidizing Fe(II). Taking advantage of this, DOXol formation and Fe(II) mobilization were measured in NADPH- and DOX-supplemented cytosols which had been reconstituted with increasing amounts of Fe(III) by incubating iron-depleted cytosols and Fe(II) for varying lengths of time. As shown in Fig. 4 *A*, DOXol decreased with increasing the cytosolic content of Fe(III). By contrast, the mobilization of Fe(II) gradually increased with increasing the Fe(III) incorporated into the cytosols (Fig. 4 *B*). These results confirmed that the enzymatic yield of DOXol was inversely related to the cytosolic content of Fe(III) and mobilization of Fe(II) (compare with Fig. 2 and Tables I and II). Therefore, we hypothesized that DOXol was consumed during the reductive mobilization of iron. This hypothesis was tested by incubating purified enzymatic DOXol with iron-reconstituted cytosols and by monitoring the fate of DOXol and the mobilization of Fe(II). As shown in Fig. 5 *A*, DOXol was quantitatively recovered from incubations with iron-depleted cytosols, but it was extensively consumed by cytosols which had been reconstituted with increasing amounts of Fe(III). The ferric iron-dependent loss of DOXol was paralleled by a stoichiometric recovery of DOX and a twofold mobilization of Fe(II) (Fig. 5 *B*). Collectively, these experiments provided evidence for a direct interaction of DOXol with Fe(III), and

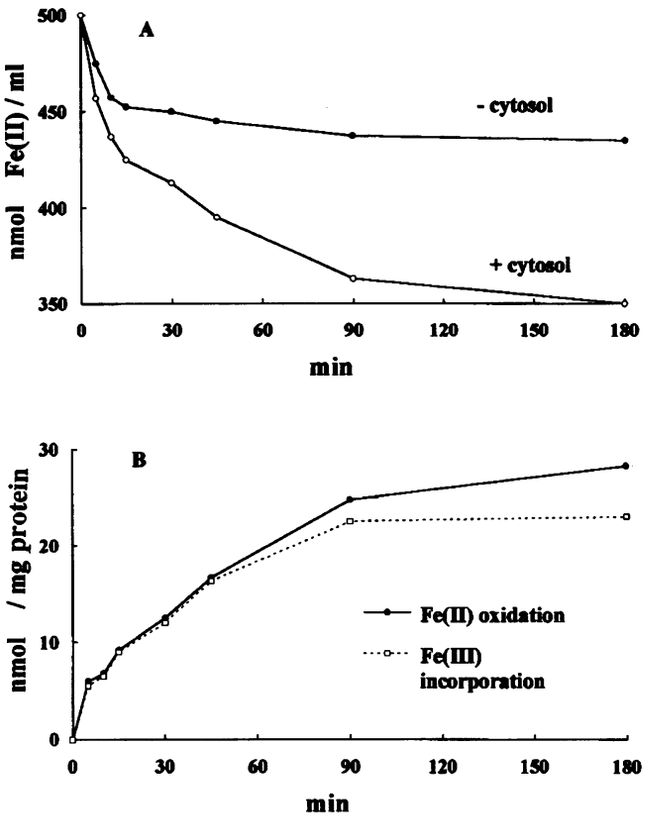


Figure 3. Fe(II) oxidation and Fe(III) incorporation by iron-depleted human heart cytosols. In (*A*) the recovery of bathophenanthroline-chelatable Fe(II) was monitored in incubations containing $FeCl_2$ plus or minus iron-depleted cytosols from postmortem samples, as described in Methods. In (*B*) the difference between cytosol-induced and spontaneous oxidation of Fe(II) was determined and compared with the incorporation of Fe(III). Values are means of two separate determinations with > 95% experimental agreement.

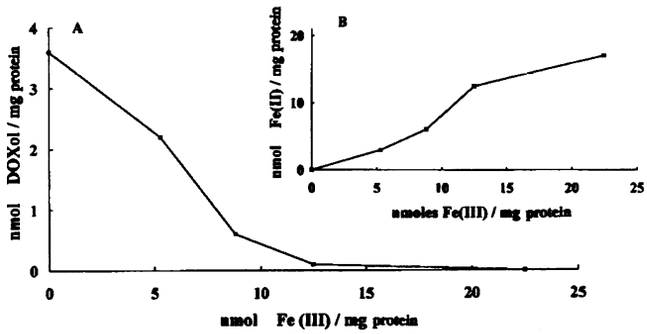
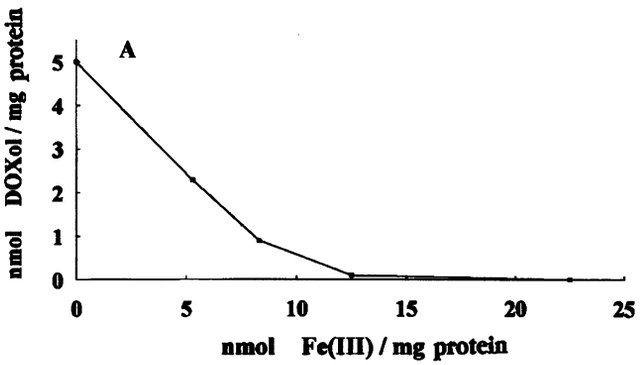
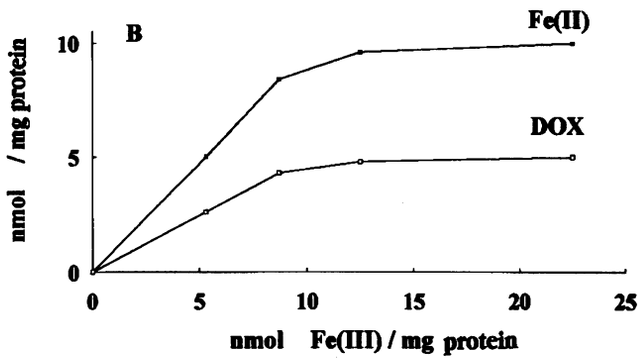


Figure 4. Doxorubicinol formation and Fe(II) mobilization in human heart cytosols with increasing Fe(III) contents. Iron-depleted cytosols from postmortem samples were incubated with $FeCl_2$ for 5, 15, 30, and 180 min to permit the incorporation of the indicated amounts of Fe(III). In (*A*) DOXol was measured in incubations containing NADPH-supplemented iron-depleted or iron-reconstituted cytosols. In (*B*) cytosols were assayed for the mobilization of Fe(II). Values are those determined at 4 h and are means of two separate determinations with 91% (*A*) or 89% (*B*) experimental agreement.





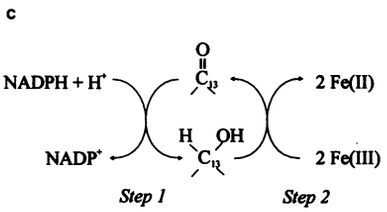


Figure 5. Doxorubicinol consumption, DOX recovery, and Fe(II) mobilization in iron-depleted or iron-reconstituted cytosols. Iron-reconstituted samples were prepared as described in the legend to Fig. 3. Incubations (1 ml final vol) contained iron-depleted or iron-reconstituted cytosols (1 mg protein), purified enzymatic DOXol (5 nmol), and bathophenanthroline disulfonate (0.25 mM) in 0.3 M NaCl, pH 7.0, 37°C. The incubations were monitored for DOXol consumption (*A*) and DOX formation or Fe(II) mobilization (*B*). Values are those determined at 1 h and are means of two separate determinations with > 95% experimental agreements. (*C*) Two-step mechanism for the mobilization of Fe(II) in NADPH- and DOX-supplemented human heart cytosols.

suggested that the overall mechanism of NADPH- and DOX-dependent Fe(II) mobilization was mediated by a twostep reaction. Step 1 consisted of an enzymatic two electron reduction of DOX to DOXol, whereas step 2 consisted of a nonenzymatic two electron reoxidation of DOXol to DOX, coupled with the reductive mobilization of two iron ions (Fig. 5 *C*). This mechanism would adequately explain how the enzymatic yield of DOXol increased or decreased upon iron removal or reincorporation, respectively.

The mechanism of iron mobilization was further investigated by incubating increasing amounts of DOXol with unmodified cytosols. As shown in Table III, purified enzymatic DOXol was capable of mobilizing Fe(II) in a concentration-dependent manner. Again, the release of Fe(II) was twice the concurrent formation of DOX, in keeping with the oxidation of one DOXol

Table III. Fe(II) Mobilization and DOX Formation in DOXol-Supplemented Unmodified Cytosols

DOXol	Fe(II) release	DOX formation	DOXol:Fe(III)	DOXol oxidation
	nmol/mg protein			%
1	2.0	1.0	0.11	100
2	3.9	2.0	0.23	100
4	7.8	4.0	0.46	100
6	8.6	4.3	0.68	72
8	8.7	4.4	0.91	55
10	8.5	4.2	1.10	42

The incubations (1 ml final vol) contained unmodified cytosols from postmortem samples (1 mg protein/8.8 nmol Fe^{III}), bathophenanthroline disulfonate (0.25 mM), and increasing amounts of purified enzymatic DOXol in 0.3 M NaCl, pH 7.0, 37°C. After spectrophotometric determination of Fe(II) release, the incubations were extracted with CH_3OH:$CHCl_3$ (1:1) to assay for DOX as an index of DOXol oxidation. In the last two columns, DOXol oxidation is given as a function of the DOXol:Fe(III) ratio in each individual incubation. Values are those determined at 1 h and are means of two separate determinations with > 95% experimental agreement.

at the expense of two Fe(III). This ~ 0.5 stoichiometry of DOXol oxidation vs Fe(III) reduction was confirmed by measuring DOXol oxidation as a function of the molar ratio with cytosolic Fe(III). In fact, DOXol oxidation decreased when the DOXol:Fe(III) ratio exceeded ~ 0.5, i.e., when the electron donor (DOXol) was in excess of the electron acceptor (Fe^{III}) (see also Table III). Similar studies with chemical DOXol gave a ≥90% experimental agreement (not shown). At this time, evidence for a ~ 0.5 stoichiometry of DOXol oxidation vs Fe(III) reduction can also be inferred from previous experiments with NADPH- and/or DOX-supplemented, unmodified, or iron-depleted cytosols. For example, the experiments with postmortem cytosols showed that NADPH or DOX per se mobilized 0.7±0.1 or 0.7±0.3 nmol Fe(II)/mg protein, respectively, whereas DOX plus NADPH mobilized 7.5±0.2 nmol Fe(II)/mg protein, according to the net increase of 6.1±0.7 nmol Fe(II)/mg protein (compare with Fig. 2). Considering that iron removal abolished Fe(II) mobilization but increased the yield of DOXol from 0.7±0.2 to 3.9±0.4 nmol/mg protein (compare with Table I), one can calculate that 3.2±0.4 nmol of DOXol were involved in the mobilization of 6.1±0.7 nmol Fe(II), in keeping with a median stoichiometry of DOXol oxidation vs Fe(III) reduction of 0.54±0.04. Similar calculations with ex vivo pre- or post-CPB samples gave stoichiometries of 0.54 or 0.53, respectively.

In other experiments with DOX-supplemented postmortem cytosols, the effects of replacing NADPH with NADH were studied. The results showed that: (*a*) iron release decreased from 6.1±1.7 to 1.8±0.3 nmol/mg protein per 4 h ($n = 4$, $P < 0.01$); and (*b*) DOXol formation was apparently abolished in unmodified cytosols and significantly decreased in iron-depleted cytosols (from 3.9±0.4 to 0.9±0.2 nmol/mg protein per 4 h; $n = 4$, $P < 0.01$). Considering that iron removal did not increase the yield of other metabolites, one can calculate that 0.9±0.2 nmol of DOXol were involved in the reductive mobilization of 1.8±0.3 nmol of Fe(II), according to a median stoichiometry of DOXol oxidation vs Fe(III) reduction of 0.51±0.03. There-

*Table IV. NADPH- and Anthracycline-dependent Fe(II) Mobilization in Unmodified Cytosols: Comparison of DOX with DNR, 4′-epi DOX, and 4-demethoxy DNR**

Anthracycline	nmol Fe(II)/mg protein per 4 h
DOX	6.1±0.7
DNR	3.5±0.6‡
4′-epi DOX	1.8±0.2‡§
4-demethoxy DNR	0.9±0.3‡‖

* NADPH- and anthracycline-supplemented unmodified cytosols from postmortem samples were assayed for Fe(II) mobilization as described in Methods. Values are those determined at 4 h and are means±SE (n = 4). ‡ Significantly different from DOX ($P < 0.01$). § $P < 0.01$ vs DNR. ‖ $P < 0.01$ vs 4′-epi DOX.

fore, the NADH-dependent mechanism of iron mobilization was stoichiometrically similar to that promoted by NADPH, however, the overall reaction was limited by a decrease of DOXol formation. This is in keeping with previous demonstration that NADH cannot substitute for NADPH as a source of reducing equivalents for anthracycline C-13 reductases (15, 16).

Doxorubicinol-dependent iron reduction is mediated by a direct electron transfer mechanism rather than by a redox coupling with oxygen and formation of $O_2^{\bullet -}$ as the ultimate reductant. In fact, DOXol oxidation did not occur upon aerobic incubation with iron-depleted cytosols (compare with Fig. 5 *A*), nor did it occur when the DOXol:Fe(III) ratio exceeded an optimum value of ~ 0.5 (compare with Table III). This means that DOXol redox couples with iron, not with oxygen. Moreover, experiments with unmodified cytosols from postmortem samples have shown that NADPH- and DOX-dependent Fe(II) mobilization occurs independently of the presence or absence of 100 U/ml of CuZnSOD (5.9±1 vs 6.3±0.8, respectively; n = 4, $P > 0.05$). Similar results have been obtained in a duplicate experiment with an ex vivo Maze's sample (6.2 [+SOD] vs 6.1 [−SOD]).

(d) Comparisons of DOX with other anthracyclines. Having demonstrated the formation of DOXol and its redox coupling with iron, we performed comparative experiments with other anthracyclines of clinical interest. Studies with NADPH- supplemented unmodified cytosols showed that DNR, 4′-epi DOX, and 4-demethoxy DNR released ~ 57, 30, and 15% of the iron released by DOX, respectively (Table IV). According to Fig. 5 *C*, iron release might be affected by a decreased formation of C-13 metabolites (*Step 1*) and/or by an altered interaction of C-13 metabolites with iron (*Step 2*). Therefore, the two steps were examined under separate experimental conditions. Step 1 was studied in iron-depleted cytosols, to abrogate redox couplings with Fe(III) and determine the net reduction of DNR, 4′-epi DOX, and 4-demethoxy DNR to the corresponding secondary alcohols. As shown in Fig. 6 *A*, DNR, 4′-epi DOX, and 4-demethoxy DNR were 46, 69, and 84% less susceptible than DOX to an enzymatic reduction at C-13. Step 2 was studied by reconstituting purified enzymatic DNRol, 4′-epi DOXol, and 4-demethoxy DNRol with unmodified cytosols, to evaluate how effectively they redox coupled with cytosolic Fe(III). As shown in Fig. 6 *B*, DNRol, 4′-epi DOXol, and 4-demethoxy DNRol were as effective as DOXol in redox coupling with Fe(III), inasmuch as they oxidized back to the parent compounds by

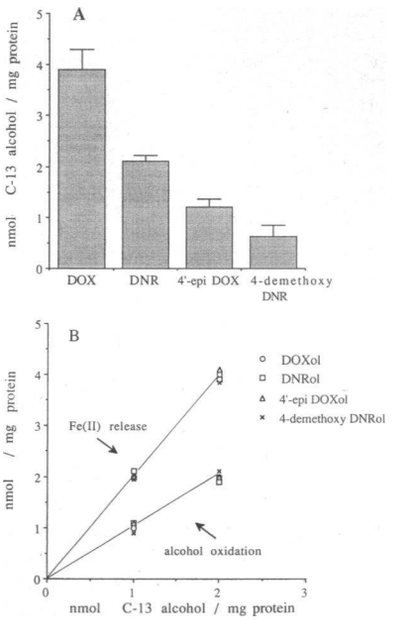


Figure 6. C-13 metabolite formation and redox coupling with iron: comparisons of DOX with DNR, 4′-epi DOX, and 4-demethoxy DNR. In (*A*) NADPH- and anthracycline-supplemented iron–depleted cytosols from postmortem samples were assayed for C-13 secondary alcohol formation. Values are those determined at 4 h and are means±1 SE (n = 4). DNRol, 4′-epi DOXol, and 4-demethoxy DNRol were significantly different from DOXol ($P <$ 0.01). 4′-epi DOXol was ($P < 0.01$) vs DNRol, and 4-demethoxy DNRol was ($P < 0.05$) vs 4′-epi DOXol. In (*B*) the incubations (1 ml final vol) contained two different concentrations of purified enzymatic alcohols, unmodified cytosols from postmortem samples (1 mg protein), and bathophenanthroline disulfonate (0.25 mM), in 0.3 M NaCl, pH 7.0, 37°C. These incubations were assayed for the release of Fe(II) and subsequently extracted with CH_3OH:$CHCl_3$ (1:1) to determine the reoxidation of alcohol metabolites back to the parent compounds. Values are those determined at 1 h and are means of two separate determinations with > 95% experimental agreement.

reductively mobilizing a twofold excess of Fe(II). Thus, a specific modification of Step 1 affected iron release by DNR, 4′-epi DOX, and 4-demethoxy DNR.

(e) Comparisons of cytosolic iron with recombinant human ferritins. Three lines of evidence showed that cytosols were essentially free of ferritin and interacted with iron by virtue of alternate Fe(III)-binding proteins. First, all the Fe(III) ions of unmodified or iron-loaded cytosols could easily pass through an affinity chromatography column which immobilized human rHF and rLF (Table V). Second, the iron bound to either cytosol

Table V. Affinity Chromatography of Ferritins and Unmodified or Iron-loaded Cytosols

Sources of iron	Sample	Tris effluent	Mg effluent
		nmol Fe(III)	
Cytosol			
Unmodified	64	63	n.d.
Iron-loaded	184	185	n.d.
rHF + rLF	120	n.d.	105

A CNBr-Sepharose 4B column with anti-rHF and anti-rLF monoclonal antibodies was charged with (*a*) an unmodified or iron-loaded cytosol from ex vivo Maze's samples, or (*b*) a mixture of self-reconstituted rHF and ceruloplasmin-reconstituted rLF, corresponding to the indicated amounts of iron. Samples were eluted with the equilibration buffer (*Tris effluent*) or 4.5 M $MgCl_2$ (*Mg effluent*). Other conditions were as described in Methods. n.d., not detectable.

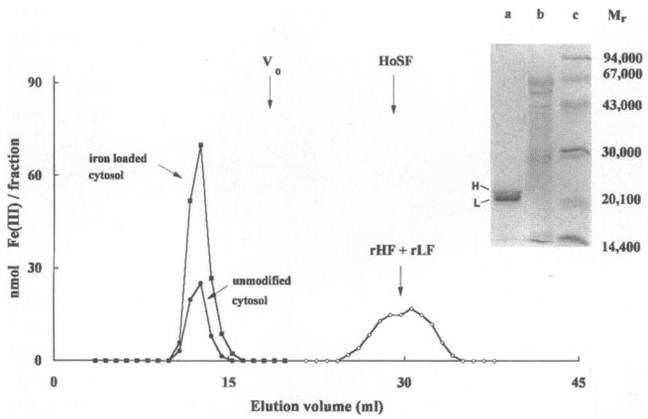


Figure 7. Sepharose 6B chromatography of cytosolic Tris effluents or ferritin Mg effluents. The Tris effluent of unmodified or iron-loaded cytosols and the Mg effluent of rHF plus rLF were chromatographed on a Sepharose 6B column and analyzed for nonheme iron distribution as described in Methods. Fractions with iron were pooled, concentrated by ultrafiltration, and subjected to SDS-PAGE analysis. The inset shows an electrophoretogram where lane *a* was loaded with 5 μg of the fractions containing rHF plus rLF, and lane *b* was loaded with 10 μg of the iron fractions from unmodified cytosol. Lane *c* was loaded with the following M_r markers: phosphorylase B (94,000); bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); and α-lactalbumin (14,400). Proteins were visualized with Coomassie brilliant blue. V_0, void volume (determined with blue dextran); HoSF, horse spleen ferritin.

preparation was excluded by a Sepharose 6B column which included rHF, rLF, and horse spleen ferritin within the same elution volume (Fig. 7). And finally, SDS-PAGE analysis showed that the Sepharose 6B fractions with the cytosolic iron lacked the $M_r \sim 20,000$ and 23,000 bands of ferritin L and H subunits, respectively (Fig. 7, *inset*). It is possible that ferritin had been immobilized by the calcium hydroxyapatite column used for increasing the stability of cytosols on freezing and thawing or reconstitution with NADPH and DOX (compare with Methods). This would be a likely consequence of strong complexation and cluster formation between the calcium sites of the resin and the acidic H subunits which predominate in heart ferritin. In the light of these results we wondered how DOXol could have reacted with cardiac isoferritins rich of H subunits. Therefore, we studied DOX formation and Fe(II) mobilization in 1-ml incubations containing 5 nmol of purified enzymatic DOXol and 10 nmol of Fe(III) bound to self- or ceruloplasmin-reconstituted rHF (corresponding to 58 or 50 μg protein, respectively). Under these conditions, neither DOX nor Fe(II) could be assayed, suggesting that DOXol did not redox couple with rHF-bound Fe(III). Negative results were also obtained by replacing rHF with equimolar iron bound to: (*a*) self- or ceruloplasmin-reconstituted rLF (corresponding to 150 or 18 μg protein, respectively); (*b*) commercially available holotransferrin (2 atoms Fe(III)/molecule, corresponding to 10 nmol Fe(III)/0.4 mg protein); or (*c*) partially (38%) saturated transferrin (0.76 atoms Fe(III)/molecule, corresponding to 10 nmol Fe(III)/1.1 mg protein). The latter had been prepared by removing iron from holotransferrin under reducing conditions and by presenting apotransferrin with a nitrilotriacetic-Fe(III) complex, according to established procedures (35). By contrast, the formation of 4.8 or 4.9 nmol DOX/h, and the mobilization of 9.5 or 9.8 nmol Fe(II)/h, were observed by incubating 5 nmol of DOXol with 10 nmol of Sepharose 6B-chromatographed Fe(III) from unmodified or iron-loaded cytosols of ex vivo Maze's samples (corresponding to 0.83 or 0.29 mg protein, respectively). These results showed that the redox coupling of one DOXol with two Fe(III) was entirely contingent on the availability of alternative sources of iron.

Discussion

In this study we have demonstrated, for the first time, that human cardiomyocytes possess the NADPH-dependent enzymatic machinery which reduces DOX to DOXol. Evidence for this activity has been obtained by reconstituting NADPH and DOX with cytosolic fractions of myocardial samples from selected postmortem donors or patients undergoing open-chest cardiac surgery. This in vitro evidence provides the conceptual framework to discuss the potential role of DOXol in the clinical settings of DOX toxicity.

Previous studies on the molecular mechanisms of DOXol toxicity have emphasized that it inhibits the ATPases of sarcolemma, sarcoplasmic reticulum, or mitochondrial membranes from dog or rabbit heart (36, 37). This inhibition was shown to occur irrespective of a simultaneous addition of iron, hence, a free radical–independent mechanism was envisioned (36). However, DOXol-inhibitable enzymes, such as the mitochondrial F_1 ATPase, have been subsequently recognized to contain loosely bound iron which catalyzes an in situ formation of hydroxyl radicals or equally reactive oxidants (38). It should also be considered that laboratory buffers (39) and membrane preparations (40) are themselves contaminated by iron ions which are liable to participate in free radical reactions upon reduction from the ferric to the ferrous form. Therefore, one can suspect that enzyme inhibition was mediated by an unrecognized free radical chemistry, involving the redox coupling of DOXol with contaminating iron. In our studies, unmodified or iron-reconstituted cytosols were prepared in NaCl and treated extensively with chelators to minimize metal contaminations, yet, they tenaciously bound an iron species that was liable to a reductive mobilization by DOXol. In light of these results, we propose that the cardiotoxicity of DOX might be the reflection of a redox coupling between DOXol and tightly bound cytosolic iron.

Enzymatic experiments with NADPH and DOX, as well as nonenzymatic experiments with purified DOXol, indicate that 1 mol of this metabolite releases 2 mol of Fe(II), i.e., the two electrons needed for reducing the C-13 carbonyl moiety of DOX are returned to two independent Fe(III) ions, according to a ~ 0.5 stoichiometry of DOXol oxidation vs Fe(III) reduction. These results point to a very efficient and novel linkage between DOX bioactivation and iron reduction, inasmuch as the DOX semiquinone free radical/$O_2^{\cdot -}$ couple would reduce < 1 Fe(III) (12). Moreover, we have shown that the redox coupling of DOXol with iron does not involve $O_2^{\cdot -}$ (compare with Results, section *C*), suggesting that SOD might not be the first-line defense against DOX toxicity. This could very well account for the finding that copper-deficient diets decrease the tissue levels of SOD activity, but fail to exacerbate the development of a cardiomyopathy in DOX-treated animals (41).

An intriguing feature of the anthracycline antibiotics is that minor chemical modifications usually result in rather major changes of cardiotoxicity. For example, DNR is in all similar

to DOX except that its side chain terminates with a methyl group in place of a primary alcohol. Nevertheless, DNR is reportedly less toxic than DOX (42, 43). Similarly, a positional change in the hydroxyl group at the C-4′ of daunosamine (compare with Fig. 1) is the sole difference between DOX and 4′-epi DOX, yet, experimental and clinical evidence suggests that 4′-epi DOX is less cardiotoxic than either DOX or DNR (44–46). And finally, deoxydemethylation of the C-4 in the tetracycline ring (compare with Fig. 1) converts DNR into a new drug which appears to be the least cardiotoxic among all clinically tested anthracyclines (6, 47). Structure activity studies in vitro have failed to demonstrate significant differences between equimolar DOX, 4′-epi DOX, DNR, and 4-demethoxy DNR with respect to the redox cycling of the quinone moiety (12, 48) and iron release from ferritin (12, 49). Some differences might nevertheless surface in vivo if anthracyclines exhibit variable pharmacokinetics and tissue distribution. For example, 4′-epi DOX is unusually susceptible to glucuronidation and is excreted more rapidly than DOX (50). The decreased cardiotoxicity of 4′-epi DOX could therefore be the obvious consequence of an accelerated elimination and reduced availability to the cardiac reductases that support one electron redox cycling of the quinone moiety and iron release from ferritin. On the other hand, 4-demethoxy DNR is less toxic than DOX, DNR, or 4′-epi DOX when assessed in vivo, however, it can be more toxic when tested in vitro on neonatal rat heart myocytes (43). Collectively, these observations would cast doubts on the significance of in vitro models of myocardial damage, suggesting that the clinical cardiotoxicity of a given anthracycline depends predominantly on pharmacokinetic factors. In contrast to this picture, our studies demonstrate that the reductive mobilization of cytosolic iron decreases gradually and significantly upon replacing DOX with equimolar DNR, 4′-epi DOX, and 4-demethoxy DNR, suggesting that a stringent relationship does exist between the severity of cardiac damage in vivo and the efficiency of iron release in vitro (compare with Table IV). By dissecting the two-step mechanism leading to the reduction of cytosolic Fe(III), we have also demonstrated that DNR, 4′-epi DOX, and 4-demethoxy DNR mobilize less Fe(II) because they are less susceptible than DOX to an enzymatic formation of secondary alcohol metabolites (compare with Fig. 6 *A*). Daunorubicinol, 4′-epi DOXol, and 4-demethoxy DNRol per se would be as effective as DOXol in redox coupling with Fe(III) (Fig. 6 *B*). Collectively, these observations indicate that a two electron reduction of the side chain C-13 moiety overshadows the one-electron reduction of the quinone group as a critical determinant of anthracycline metabolism and iron mobilization, irrespective of pharmacokinetic differences that may exist between various analogs.

In an attempt to elucidate the molecular mechanisms underlying the different toxicity of DOX vs DNR, Zweier et al. (51) have suggested that a key role might be played by the side chain C-14 moiety. According to these authors, the C-14 primary alcohol of DOX oxidizes at the expense of Fe(III), yielding an aldehyde plus Fe(II) ions which subsequently catalyze free radical reactions (51). This would explain how DOX is more toxic than DNR or any DNR analog having a C-14 methyl moiety in place of a primary alcohol. Our results do not provide any evidence for the involvement of the C-14 moiety in the reductive mobilization of cytosolic iron. In fact, DOX per se could mobilize as little as one tenth of the iron mobilized upon simultaneous addition of NADPH and enzymatic formation of DOXol (compare with Fig. 2). Moreover, we have shown that cytosolic Fe(III) can be reduced to a comparable extent by DOXol, DNRol, 4′-epi DOXol, and 4-demethoxy DNRol, i.e., the C-13 metabolites of anthracyclines which either lack or contain a primary alcohol at C-14. We have no explanation for the exclusive role of the C-13 moiety in Fe(III) reduction. We can only note that the primary alcohol of DOX has been shown to reduce a low-mol-wt acetohydroxamate-Fe(III) complex (51), whereas our studies indicate that C-13 secondary alcohols reduce an iron species which is tightly bound to proteins. It is possible that the access to protein-bound iron is limited by sterical requirements that are met by a secondary alcohol at C-13, but not by a primary alcohol at C-14. We should also emphasize that the intracellular concentration of low molecular weight iron is extremely low and difficult to quantify (1, 2, 52). Hence, a reaction between secondary alcohols and the more abundant protein-bound iron should predominate as a mechanism of cardiotoxicity.

The iron-detoxifying activity of ferritin is the result of a functional cooperation between H and L subunits. The oxygen-dependent oxidation of Fe(II) occurs predominantly at discrete sites of the H subunits, whereas the size of the cavity which accommodates Fe(III) usually increases with the number of L subunits (3). Heart ferritin is composed primarily of H subunits, hence, it is particularly well suited for the rapid sequestration of iron. By contrast, liver and spleen ferritins are composed primarily of L subunits and are more suitable for the long-term storage of copious amounts of iron (3, 4). We have confirmed the existence of these distinct functions for H or L subunits. In fact, H-type recombinant homopolymers were found to oxidatively incorporate greater amounts of iron than did L-type homopolymers devoid of ferroxidase activity (95 vs 32 atoms Fe^{III}/molecule per h). However, this difference was reverted by reconstituting ferritins in the presence of ceruloplasmin, which also oxidizes Fe(II) and is frequently used in vitro to incorporate Fe(III) into L-type apoferritins with little ferroxidase activity (2, 53, 54). In the presence of ceruloplasmin, L-type homopolymers could incorporate much greater amounts of Fe(III) than did H-type homopolymers (260 vs 110 atoms Fe^{III}/molecule per h), in keeping with a major role for L subunits in the formation of an iron core. In our study, iron-depleted cytosols were shown to oxidize Fe(II) and incorporate Fe(III) in a large excess of that recovered in unmodified samples, in apparent agreement with an H-type ferroxidase activity (compare with Figs. 3, *A* and *B* and 7). Nevertheless, we have provided analytical and functional evidence that: (*a*) iron was oxidatively incorporated by proteins distinct from ferritin (compare with Fig. 7 and Table V); and (*b*) ferritin did not redox couple with DOXol, irrespective of the subunit composition, the iron:protein ratio, and the presence or absence of ceruloplasmin at the time of iron incorporation into apoferritin (compare with Results, section *e*). Purified DOXol was also shown not to redox couple with transferrin, irrespective of the degree of iron saturation (see Results, section *e*). Hence, the DOXol-releasable iron was not accounted for by transferrin which had been released from endocytic vesicles during tissue manipulation and cytosol preparation. These results indicate that the availability of nonferritin sources of Fe(III) represents an additional determinant of the molecular linkage between DOX metabolism and Fe(II) mobilization. The existence of "nonferritin" pools of iron, having variable mol wt and intracellular distribution, has been previously postulated by several research groups (55–60). The

precise nature and biologic significance of these sources of iron have remained unknown, mostly because of technical difficulties in achieving purification and molecular characterization (55, 60). It has been tentatively proposed that the nonferritin pools of iron may serve, upon metabolic needs, for the synthesis of hemeproteins (60). Alternatively, these pools of iron might identify with enzymes which operate by virtue of essential nonheme Fe(III) moieties (61). In either case, DOXol-dependent delocalization of these sources of iron would interrupt important cell functions, irrespective of any subsequent involvement of delocalized Fe(II) in free radical reactions and pharmacologic intervention with iron chelators. This scenario might very well provide an additional explanation for the incomplete cardioprotection afforded by dexrazoxane.

In vitro studies with tumor cell lines indicate that anthracycline C-13 metabolites have much less tumoricidal activity than have the parent compounds, with the possible exception of 4-demethoxy DNRol being equipotent as 4-demethoxy DNR (37, 62). On the other hand, our studies indicate that an intramyocardial mobilization of Fe(II) depends very critically on the formation of C-13 metabolites which reduce Fe(III). This mechanistic dissociation, as well as the clinical need for "better" anthracycline regimens and cardioprotective interventions (6), would possibly identify inhibitors of anthracycline C-13 reductases as a new class of drugs that mitigate cardiotoxicity without affecting antitumor activity. The purification and characterization of intracardiac C-13 reductases would help to select potential inhibitors and accelerate their in vivo testing as cardioprotectants.

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