Paradoxical Inhibition of Cardiac Lipid Peroxidation in Cancer Patients Treated with Doxorubicin

Pharmacologic and Molecular Reappraisal of Anthracycline Cardiotoxicity

Giorgio Minotti,* Cesare Mancuso,* Andrea Frustaci,‡ Alvaro Mordente,§ Stefano A. Santini,§ Antonio Maria Calafiore,‖ Giovanni Liberi,‖ and Nicolo' Gentiloni‡

*Departments of *Pharmacology, ‡Medicine, and §Biochemistry, Catholic University School of Medicine, 00168 Rome; and ‖Department of Cardiac Surgery, G. D'Annunzio University School of Medicine, 66100 Chieti, Italy*

Abstract

Anticancer therapy with doxorubicin (DOX) and other quinone anthracyclines is limited by severe cardiotoxicity, reportedly because semiquinone metabolites delocalize Fe(II) from ferritin and generate hydrogen peroxide, thereby promoting hydroxyl radical formation and lipid peroxidation. Cardioprotective interventions with antioxidants or chelators have nevertheless produced conflicting results. To investigate the role and mechanism(s) of cardiac lipid peroxidation in a clinical setting, we measured lipid conjugated dienes (CD) and hydroperoxides in blood plasma samples from the coronary sinus and femoral artery of nine cancer patients undergoing intravenous treatments with DOX. Before treatment, CD were unexpectedly higher in coronary sinus than in femoral artery (342±131 vs 112±44 nmol/ml, mean±SD; $P < 0.01$), showing that cardiac tissues were spontaneously involved in lipid peroxidation. This was not observed in ten patients undergoing cardiac catheterization for the diagnosis of arrhythmias or valvular dysfunctions, indicating that myocardial lipid peroxidation was specifically increased by the presence of cancer. The infusion of a standard dose of 60 mg DOX/m^2 rapidly (∼ 5 min) abolished the difference in CD levels between coronary sinus and femoral artery (134±95 vs 112±37 nmol/ml); moreover, dose fractionation studies showed that cardiac release of CD and hydroperoxides decreased by ∼ 80% in response to the infusion of as little as 13 mg DOX/m^2. Thus, DOX appeared to inhibit cardiac lipid peroxidation in a rather potent manner. Corollary in vitro experiments were performed using myocardial biopsies from patients undergoing aorto-coronary bypass grafting. These experiments suggested that the spontaneous exacerbation of lipid peroxidation probably involved preexisting Fe(II) complexes, which could not be sequestered adequately by cardiac isoferritins and became redox inactive when hydrogen peroxide was included to simulate DOX metabolism and hydroxyl radical formation. Collectively, these in vitro and in vivo studies provide novel evidence for a possible inhibition of cardiac lipid peroxidation in DOX-treated patients. Other processes might therefore contribute to the cardiotoxicity of DOX. (*J. Clin. Invest.* 1996. 98:650–661.) Key words: doxorubicin • iron • free radicals • lipid peroxidation • cardiotoxicity

Introduction

The clinical usefulness of doxorubicin (DOX)[1] and other anticancer anthracyclines is limited by peculiar toxicities to cardiac tissues. A digitalis-unresponsive congestive heart failure has been documented in greater than 30% of patients after treatments with cumulative doses above 600 mg/m^2 (1). However, severe dysfunctions may also occur in response to cumulative doses below 400 mg/m^2, especially when DOX is given in combination with other chemotherapeutic agents (2). Doxorubicin-treated patients are therefore at risk for acute and chronic cardiotoxicities, imposing dose restrictions and surveillance of the cardiovascular performance.

Previous attempts to elucidate the molecular mechanism(s) of cardiac damage have emphasized the role of a quinone moiety that is placed in the tetracycline ring of DOX and participates in reduction-oxidation processes. In fact, in vitro studies have shown that mitochondrial, nuclear and microsomal NAD(P)H oxidoreductases catalyze a one-electron reduction of the quinone group of DOX, yielding a semiquinone free radical that regenerates the parent compound by oxidizing with molecular oxygen (3). It follows that DOX administration exposes tissues to substantial fluxes of $O_2^{\cdot-}$ and H_2O_2. According to the prevailing hypothesis, cells with high levels of $O_2^{\cdot-}$ and H_2O_2-detoxifying enzymes resist the perturbing effects of DOX metabolism, whereas cells with low levels are expected to succumb. The latter should be the case for cardiomyocytes, as they contain less catalase, glutathione peroxidase, and superoxide dismutase than other cells (3–6).

The cytotoxicity of $O_2^{\cdot-}$ and H_2O_2 is substantially enhanced by iron. For example, the reaction of H_2O_2 with the heme iron moiety of myoglobin generates an oxoferryl species that promotes lipid peroxidation (7). While deficient in H_2O_2-detoxifying enzymes, cardiomyocytes are very rich in myoglobin; hence, they may represent the ideal scenario for a pathologic process of heme iron-dependent lipid peroxidation. Hydrogen peroxide can also promote lipid peroxidation through the reaction with low mol wt Fe(II) complexes, yielding hydroxyl radicals (8). Under physiologic conditions, this alternative mechanism of H_2O_2 toxicity is precluded by the presence of ferritin, a

Address correspondence to Dr. Giorgio Minotti, Department of Pharmacology, Catholic University School of Medicine, Largo F. Vito 1, 00168 Rome, Italy. Phone: 39-6-30154367; FAX: 39-6-3050159.

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1. *Abbreviations used in this paper:* CD, conjugated dienes; CS, coronary sinus; DOX, doxorubicin; FA, femoral artery; H, heavy; L, light; LOOH, lipid hydroperoxides; rHF, recombinant heart-type ferritin homopolymer; rLF, recombinant liver-type ferritin homopolymer; TBA, thiobarbituric acid; TBARS, thiobarbituric acid–reactive substances.

Table I. Tumor Parameters and Individual Risk Factors of Patients Included in the Lipid Peroxidation Study

Patient	Age	Sex	Tumor	Stage	Individual risk factors
1	44	F	Retroperitoneal fibrosarcoma	IV	None
2	67	F	Ovary adenocarcinoma	IV	None
3	19	M	Non-Hodgkin's lymphoma	III-B*	None
4	29	M	Non-Hodgkin's lymphoma	II-A*	None
5	48	M	Non-Hodgkin's lymphoma	IV-B*	None
6	53	M	Urothelial bladder carcinoma	IV	None
7	29	M	Non-Hodgkin's lymphoma	II-B*	Irradiation of mediastinum
8	43	F	Ovary undifferentiated carcinoma	IV	None
9	39	M	Epithelial type peritoneal mesothelioma	IV	None

*A, asymptomatic patient; B, patients with history of fever, sweats, or weight loss > 10%.

24-subunit cytosolic protein that decreases the levels of low mol wt Fe(II) by virtue of a "ferroxidase activity" coupled with the incorporation of Fe(III) (9). This protective mechanism would nevertheless be breached as the continuous reduction and oxidation of DOX exceeds the superoxide dismutase activity of cardiomyocytes, thus allowing $O_2^{\cdot-}$ to delocalize Fe(II) by reducing ferritin-bound Fe(III) (10).

Electron spin resonance techniques have demonstrated the formation of hydroxyl radicals in DOX-perfused rat heart preparations, confirming that this drug can overwhelm the intracardiac defenses against reactions between H_2O_2 and iron (11). The pathophysiologic implications of these processes have nevertheless remained unclear. In fact, studies in DOX-treated laboratory animals have produced both positive (12, 13) and negative (14–17) evidence for a cause–effect relationship between lipid peroxidation and cardiovascular dysfunctions. This experimental divergence would provide a rationale to evaluate cardiac lipid peroxidation under clinical conditions; however, such a study is made difficult by both ethical and practical reasons, precluding fine needle myocardial biopsies of sufficient size and number to perform biochemical analyses. We have therefore designed an alternative procedure, involving catheterization of the coronary sinus and measurements of lipid peroxidation products that cardiac tissues may release therein. This procedure has enabled us to unravel a free radical paradox, in the sense that cancer patients have a spontaneous exacerbation of cardiac lipid peroxidation that is abolished by DOX treatments. By combining these observations in vivo with ancillary experiments in vitro, we have also been able to show that: (*a*) the spontaneous enhancement of lipid peroxidation may involve preexisting low mol wt iron complexes that are not adequately sequestered by cardiac isoferritins; and (*b*) the reaction of iron with lipids ceases in the presence of a stoichiometric excess of H_2O_2, as it presumably occurs upon DOX administration and semiquinone formation. Collectively, these findings appear to dispel the role and mechanisms of lipid peroxidation in cardiac damage, setting the stage for a critical reappraisal of the experimental and clinical aspects of DOX toxicity.

Methods

In vivo studies

Patients. Cardiac lipid peroxidation was studied in patients who met the following inclusion criteria: (*a*) histologic diagnosis and clinical staging of tumors with a documented sensitivity to DOX; (*b*) electrocardiographic, x-ray, and ultrasound evidence for normal cardiac morphology and functions; (*c*) performance status ≥ 60%, according to Karnofsky's grading (18); (*d*) hemoglobin ≥ 8 g/dl; and (*e*) no previous treatment with anthracyclines or other anticancer drugs. Major exclusion criteria involved cigarette smoking and high plasma cholesterol (> 200 mg/dl) or triglycerides (> 170 mg/dl), as these conditions accompany with anomalous increases of the plasma levels of lipid peroxidation products (19–21). Table I summarizes tumor parameters and individual risk factors of nine consecutive patients (six M and three F, aged 41.2±14 yr) who met the requirements for study entry. One of these patients had been previously subjected to mediastinum irradiation as a part of a combined treatment for a large mass of non-Hodgkin's lymphoma. Thoracic irradiation increases the chances to develop cardiac damage upon subsequent treatments with anthracyclines (2). Therefore, this patient had an individual risk of cardiotoxicity and was scheduled for treatments with lower doses of DOX (see Tables I and III, and Results).

Treatments, blood sampling, and lipid peroxidation measurements. After an overnight fast, patients were placed under electrocardiographic monitoring and subjected to femoral artery and coronary sinus catheterization. The right femoral artery was entered by Seldinger's technique (22) and catheterized by a 6F sheath suitable for blood sampling. Coronary sinus was catheterized by percutaneous left basilic vein approach. Briefly, a 7F Zucker catheter (USCI-BARD, Billerica, MA) was advanced under fluoroscopic monitoring and placed in the middle portion of coronary sinus with sufficient clearance to allow for free aspiration of blood. Unless otherwise indicated, ~ 1–2-ml blood samples were drawn from catheters into heparinized Vacutainer Tubes (Becton Dickinson, Inc., Rutherford, NJ) before and 5 min after the rapid (~ 3 min) infusion of DOX (Adriblastina®, Pharmacia-Farmitalia Carlo Erba, Milan, Italy). Similar samples were collected from antecubital veins. Throughout this treatment, there were no relevant electrocardiographic abnormalities, including rhythm disturbances or alterations of the repolarization phase. For comparative purposes, blood samples were also collected from the femoral artery and coronary sinus of eight normolipidemic patients (five M and three F, 28±4 yr) undergoing cardiac catheterization for the electrophysiologic characterization of Wolff-Parkinson-White disease, a supraventricular arrhythmia. Additional samples were obtained from two normolipidemic female patients, 50 and 59 yr, undergoing hemodynamic evaluation of mitral valve prolapse or stenosis, respectively. This general protocol, as well as the modifications shown later in Table III and Fig. 3, had been approved by the Committe for Ethics in Clinical Research at the Catholic University School of Medicine (Rome, Italy). Informed consent was obtained from each patient before DOX treatment and/or blood sampling.

After a 15-min centrifugation of the blood samples at 1,500 *g*, 4°C, plasma was aspirated and extracted immediately as described by

Folch et al. (23). The organic phases were recovered and dried under vacuum in a Speed-Vac concentrator (RC 100; Savant Instruments Inc., Farmingdale, NY). Dry residues were eventually assayed for lipid peroxidation by measuring conjugated dienes (CD) and lipid hydroperoxides (LOOH). For CD measurements, samples were diluted as appropriate in spectroscopic-grade cyclohexane or methanol (Pharmacia-Farmitalia Carlo Erba) and identified from their typical adduct at 234–240 nm using a diode array spectrophotometer (8452A; Hewlett Packard Co., Waldbronn, Germany). After computer-assisted corrections for scattering and background absorbance due to unperoxidized lipids, CD were quantified by assuming $\epsilon_{234\ nm} = 28\ mM^{-1}\ cm^{-1}$ (24). Where indicated, CD were further characterized by second-derivative spectroscopy (25, 26). Lipid hydroperoxides were measured by the ferrous-xylenol orange assay (27), with modifications for studies in plasma samples (28). Briefly, vacuum-dried plasma extracts were suspended in 0.1 ml of methanol and incubated for 30 min at room temperature with 0.9 ml of a reagent mixture composed of 0.25 mM $(NH_4)_2FeSO_4$, 0.1 mM xylenol orange [*o*-cresolsulfonphthalein-3,3′-bis(methyliminodiacetic acid)sodium salt], 25 mM H_2SO_4, and 4 mM butylated hydroxytoluene in 90% (vol/vol) methanol. Under these conditions, LOOH oxidize Fe(II) to Fe(III), which is chelated by xylenol orange to form a chromogenic complex with a spectral maximum at 560 nm ($\epsilon = 15\ mM^{-1}\ cm^{-1}$) (27). Lipid hydroperoxides were routinely quantified by assuming $\epsilon_{560\ nm} = 45\ mM^{-1}\ cm^{-1}$, inasmuch as 1 mol LOOH can oxidize 3 mol Fe(II) (28). For the purpose of method validation, dry residues from each plasma sample were also suspended in 0.1 ml of methanol-dissolved triphenylphosphine (1 mM), which selectively reduces LOOH to hydroxy derivatives having no reactivity with Fe(II) (28, 29). After a 30-min incubation at room temperature, triphenylphosphine-treated samples were reacted for an additional 30 min with 0.9 ml of the xylenol orange/Fe(II) reagent, and shown not to produce spectrally measurable ferric complexes. This confirmed that the method was specific for the hydroperoxide moieties of authentic LOOH (28). Control experiments also showed that neither ferritin, nor transferrin or low mol wt iron complexes (e.g., ADP-Fe[II]) were partitioned into the organic phase of Folch's extractions. Therefore, biologic sources of iron, possibly present in unextracted plasma samples, did not interfere with xylenol orange or Fe(II) during the assay for LOOH in Folch's extracts. All chemicals for LOOH measurements were from Sigma Chemical Co. (St. Louis, MO), with the exception of xylenol orange, which was purchased from Aldrich Chemical Co. (Milwaukee, WI).

In vitro studies

Materials. Ammonium sulfate (ultrapure grade, Fe(III) < 0.5 ppm) was purchased from Schwarz/Mann (Cleveland, OH). EDTA and $FeSO_4$ were from Merck (Darmstadt, Gemany), whereas H_2O_2 and L-histidine were from Aldrich Chemical Co. and Calbiochem-Behring Corp. (La Jolla, CA), respectively. 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) was a product of Polysciences, Inc. (Warrington, PA). Bovine liver catalase (EC 1.11.1.6) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany) and was made free of thymol by ultrafiltration in Diaflo membranes (YM100; Amicon, Inc., Beverly, MA). Electrophoresis reagents were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); all other chemicals were obtained from Sigma Chemical Co. Electrophoretically homogeneous recombinant heart-type and liver-type ferritin homopolymer (rHF and rLF, respectively) were obtained through the courtesy of Dr. Paolo Arosio (Department of Biotechnology, Istituto Scientifico San Raffaele, Milan, Italy). Ferritin expression and purification were as described by Levi et al. (30). 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 (8, 31, 32). Although unbuffered, the pH of the reaction mixtures did not vary throughout the experiment time. All the solutions were prepared with double-distilled water that had been passed through a Milli-Q Water System (Millipore Corp. Marlborough, MA). Trace metals were eventually removed by ion-exchange chromatography on Chelex 100 (Bio Rad Laboratories, Richmond, CA).

Lipid peroxidation experiments. Aliquots of ethanol-dissolved sodium arachidonate were vacuum-dried, suspended in 0.3 M NaCl, pH 7.0, and subjected to vesicle formation by indirect anaerobic sonication (33). Lipid peroxidation was studied in incubations (0.5–1 ml final vol) containing arachidonic acid vesicles (0.4 mM) and either $FeSO_4$ (0.1 mM) or metmyoglobin (0.2 mM). The latter had been previously quantified by assuming $\epsilon_{630\ nm} = 3.5\ mM^{-1}\ cm^{-1}$ (7). Where indicated, ADP or L-histidine were included to achieve a 10:1 ratio with $FeSO_4$. This was done because the intracellular formation of low mol wt iron complexes is thought to involve a stoichiometric excess of oxygen or nitrogen donor ligands such as ADP or histidine, respectively (34). Where indicated, increasing amounts of H_2O_2 were also included to simulate DOX treatments and reaction of semiquinone metabolites with oxygen. After 30-min incubation at 37°C, lipid peroxidation was measured as the formation of thiobarbituric acid–reactive substances (TBARS), according to Buege and Aust (35) with modifications involving butanol extraction of the thiobarbituric acid (TBA) adduct (36). The antioxidant butylated hydroxytoluene (0.03 vol in 2% ethanol) was added to the TBA reagent to prevent further peroxidation of lipids during the heating step of the assay (35). Before the TBA test, all lipid peroxidation samples were treated with excess catalase (400 U/ml) for $\sim$ 30 s. This was done to ensure rapid decomposition of unreacted H_2O_2 and prevent its possible interference with the TBA test (37). In other experiments, the reaction mixtures were similarly treated with catalase and extracted with a fourfold excess of chloroform/methanol (2:1) to assay for CD and LOOH, as already described.

Isolation and oxidation of LDL. After an overnight fast, blood was collected from the antecubital veins of normolipidemic volunteers and processed for plasma separation. Low density lipoprotein was subsequently isolated by a 60-min ultracentrifugation at 362,500 *g* in a vertical rotor (Centrikon TVF 65.13; Kontron Instruments, Everett, MA), using a discontinuous density gradient obtained by overlayering 48% (wt/vol) sucrose (2 ml), 0.4 M NaCl-fortified plasma (3 ml), and 0.67 M NaCl (5 ml) (38, 39). After recovery from the mid-upper part of the gradient and dialysis against phosphate-buffered saline, pH 7.3, LDL (0.1 mg protein/ml) was incubated in the same medium and oxidized by exposure to peroxyl radicals, generated via thermal decomposition of 5 mM AAPH (40). After a 2-h reaction at 37°C, LDL was extracted with chloroform/methanol and assayed for CD and LOOH.

Preparation of cytosols from cardiac and hepatic biopsies. Small atrial samples ($\sim$ 0.2 g) were taken from 41 male and female patients, 62±12 yr, undergoing aorto-coronary bypass grafting. All biopsies were collected before cardiopulmonary bypass, using a previously described technique (41). Larger liver samples (4–5 gm) were obtained from one male and two female patients (47 to 65 yr) undergoing surgical removal of benign hepatocellular adenomas. Tissues were excised from areas surrounding the adenomas and were judged as microscopically free of inflammatory reactions or athrophic degeneration by two independent pathologists. Pooled myocardial biopsies or individual liver specimens were processed for cytosol preparation by sequential homogenization, ultracentrifugation, and 65% ammonium sulfate precipitation of 105,000 *g* supernatants, as described previously (41). In the present study, hydroxyapatite chromatography of ammonium sulfate precipitates was omitted, as it would have removed ferritin (41). Cytosols were dialyzed overnight 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) (41). Informed consent was obtained from all patients undergoing tissue sampling.

Gel filtration analysis of cytosolic iron. 8 mg protein of cytosolic fractions were concentrated to 1 ml by ultrafiltration in 10-kD exclusion limit Minicon Cells (Danvers, MA) and loaded onto a (1.8 × 27 cm) Sepharose 6B column, previously equilibrated with 0.3 M NaCl,

pH 7.0, and calibrated with blue dextran to determine the void volume (17.1 ml). Samples were eluted with the equilibration medium at the flow rate of 0.65 ml/min and 0.9-ml fractions were collected in 1.5-ml minifuge tubes containing 30 μl of 15 mM bathophenanthroline disulfonate, which forms a chromogenic complex with Fe(II), having a spectral maximum at 534 nm (ϵ = 22.14 mM^{-1} cm^{-1}) (42). All fractions were assayed for bathophenanthroline-Fe(II) before and after the addition of thioglycolic acid (0.5% vol/vol), which reduces Fe(III) to Fe(II) (41). The difference between [(bathophenanthroline-Fe(II)) plus thioglycolate] and [(bathophenanthroline-Fe(II)) minus thioglycolate] gave a measurement of Fe(III). Ferritin-bound iron was identified within the elution volume delimited by rHF and rLF (27.9 and 30.6 ml, respectively). Iron fractions excluded in the void volume were referred to as nonheme–nonferritin (41). In selected experiments, cytosols (8 mg protein/ml) had been previously reconstituted with ADP-Fe(II) (1 mM chelator and 0.1 mM $FeSO_4$) in 0.3 M NaCl, pH 7.0. After a 30-min incubation at 37°C, 1.5 mM EDTA was included to partition iron from ADP, as well as to remove iron loosely bound to the surface of cytosolic protein(s). The mixtures were stirred on ice for an additional 30 min and subsequently chromatographed on Sepharose 6B to remove EDTA-iron and analyze cytosolic proteins for changes in iron content, distribution, and redox state.

Immunoaffinity chromatography. Cytosols (6–8 mg protein/ml) were chromatographed on a (1.3 × 2 cm) CNBr-Sepharose 4B column, equilibrated with 0.15 mM NaCl and 10 mM Tris HCl, and conjugated with 1 mg of anti–rHF monoclonal RHO2 IgG_1 plus 1 mg of anti–rLF monoclonal L03 IgG_{2b} (41). Samples were eluted with the equilibration buffer, whereas column-immobilized ferritins were collected separately in 0.8-ml fractions by nondenaturing elution with $MgCl_2$ (41). We have previously shown that both ferritin immobilization and elution occur with greater than 95% efficiency (41); hence, this procedure had the dual advantage of providing ferritin-free cytosols as well as Mg eluants in which ferritin could be measured by titration with bicinchoninic acid (Pierce, Oud-Beijerland, The Netherlands), using the enhanced sensitivity protocol (43). This could obviate the limitations of enzyme- and radioimmunoassays, affecting comparisons of isoferritins with different subunit composition and immunologic architecture, as in the case of cardiac vs hepatic samples (44). Where indicated, immuno-chromatographed cytosols and ferritins were dialyzed against 0.3 M NaCl and used for lipid peroxidation experiments.

Other assays. Cytosol and LDL proteins were measured by the Lowry's method (43). SDS-PAGE under reducing conditions was performed as described by Laemmli (45), using 15% polyacrylamide gels. Spontaneous or cytosol-induced ADP-Fe(II) oxidation was measured as the loss of bathophenanthroline-chelatable Fe(II) (41). Unless otherwise indicated, all data are expressed as the arithmetic mean±SD. Statistical analyses were performed by paired and unpaired Student's t test, and differences were considered significant when $P < 0.05$. Other conditions are indicated in the figures and tables.

Results

Inhibition of cardiac lipid peroxidation by 60 mg DOX/m^2. Lipid peroxidation generates CD that convert into LOOH upon oxygen incorporation and can be found in the plasma of diseased individuals. Under physiologic conditions, however, plasma contains dietary CD that convert scarcely into LOOH and should not be viewed as biochemical indexes of endogenous lipid peroxidation. This is the case for 9-*cis*, 11-*trans* linoleic acid, which is absorbed preformed by the small intestine or is synthesized endogenously by unidentified hydrogenase(s) and isomerase(s) acting on 9-*cis*, 12-*trans* linoleic acid (46). Keeping this in mind, nine cancer patients elected to DOX treatments (Table I) were sampled for circulating CD and LOOH in comparison with sex- and age-matched healthy subjects (six M and three F, 37.3±13 yr). As shown in Table II, CD, LOOH, and LOOH/CD ratios were significantly higher in plasma samples from the antecubital veins of cancer patients than in similar samples obtained from healthy subjects, showing that differences existed between the two groups with respect to the levels of CD and the ease with which they converted into LOOH. We therefore used second-derivative spectroscopy to resolve the absolute spectrum of CD in minima that can be seen at ~ 230 and/or ~ 240 nm, depending on the biologic environment(s) in which lipids have been oxidized (25, 26, 47). As shown in Fig. 1 *A*, LOOH-poor CD from healthy volunteers exhibited a spectral pattern with minima at 227 and 244 nm. Biological 9-*cis*, 11-*trans* linoleic acid is not available; however, previous studies with equivalent 9-*trans*, 11-*trans* stereoisomers have demonstrated that minima at ~ 230 and ~ 240 nm are indeed indicative of the presence of large amounts of 9-*cis*, 11-*trans* linoleic acid in plasma (26). By contrast, Fig. 1 *B* shows that LOOH-rich CD from cancer patients exhibited a second-derivative spectrum with a single minimum at 244 nm, the same as observed in laboratory animals that form membrane CD and LOOH in response to oxidant injury (47). Oxidative modifications of LDL did not contribute to the spectral characteristics of CD in cancer patients. In fact, LDL that had been oxidized in vitro and contained as many as 214 or 44 nmol CD or LOOH/mg protein, respectively, exhibited a single minimum at 232 nm rather than at 244 nm (Fig. 1 *C*). Collectively, these comparative analyses showed two major modifications in cancer patients: (*a*) an increase of membrane lipid peroxidation, causing the intravascular release of LOOH-rich CD; and (*b*) a decrease or disappearance of LOOH-poor dietary CD, reflecting disturbances of the absorption, synthesis, and/or clearance of these compounds.

Following these characterizations, CD were measured in the coronary sinus and femoral artery of cancer patients before and after a bolus of DOX (60 mg/m^2 i.v.). Before DOX infusion, CD were significantly higher in coronary sinus than in

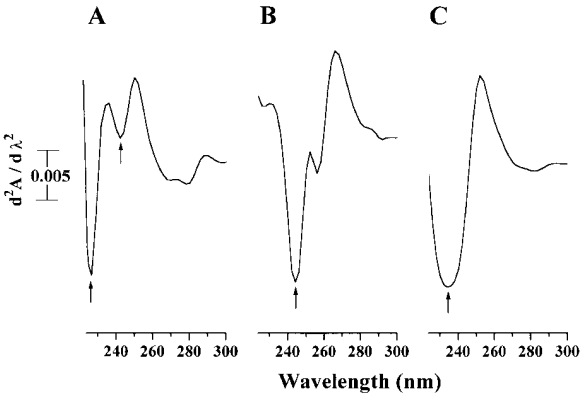

Figure 1. Second-derivative spectroscopy of CD from healthy individuals, cancer patients, and oxidized LDL. All CD samples had been diluted to achieve ~ 0.6 U of absorbance at 234 nm and permit direct comparisons. *A* is the second-derivative spectrum of plasma CD from a 39-yr-old healthy female, whereas *B* is the spectrum of a similar sample from a 43-yr-old female cancer patient (No. 8 in Table I). *C* is the spectrum of CD from LDL previously oxidized in vitro. All conditions were as described in Methods and Results. $d^2A/d\lambda^2$, second-derivative of absorbance vs wavelength.

Table II. Plasma Levels of CD and LOOH in Cancer Patients and Healthy Subjects

Sample donor	CD	LOOH	LOOH/CD
	nmol/ml		
Cancer patients	124±44*	11.2±1.5*	0.09±0.01*
Healthy subjects	55±7	1.8±0.7	0.03±0.02

CD and LOOH were measured in plasma samples from cancer patients and healthy individuals, as described in Methods and Results.
*Significantly different from healthy subjects ($P < 0.01$).

femoral artery or antecubital veins (342±131 vs 112±44 and 119±39 nmol/ml, respectively; $P < 0.01$). Conjugated dienes from femoral artery or coronary sinus had the same second-derivative spectrum as CD from antecubital veins, involving a single minimum at 244 nm (not shown). Thus, the peroxidative tone of cancer patients was specifically enhanced at the cardiac level. After DOX infusion, the CD content of samples from femoral artery and antecubital veins remained unchanged (112±37 and 131±54 nmol/ml, respectively). By contrast, DOX infusion significantly decreased the levels of CD in the coronary sinus (from 342±131 to 134±95 nmol/ml, $P < 0.01$) (Fig. 2 *A*). Having measured the individual changes of CD in femoral artery (FA) and coronary sinus (CS), we could calculate $[CD]_{CS}/[CD]_{FA}$ ratios as additional indexes of lipid peroxidation. Before DOX infusion, the $[CD]_{CS}/[CD]_{FA}$ ratios largely exceeded unity (3.4±1.6) as cardiac tissues were involved in lipid peroxidation and released CD in excess of those present in the coronary affluents. After DOX infusion, the $[CD]_{CS}/[CD]_{FA}$ ratios decreased to 1.1±0.3, indicating that cardiac lipid peroxidation had been abolished (Fig. 2 *B*). Spontaneous exacerbation of cardiac lipid peroxidation was a rather unique feature of cancer patients. In fact, the $[CD]_{CS}/[CD]_{FA}$ ratios always averaged unity in cancer-free patients with cardiac arrhythmias or valvular dysfunctions (Fig. 2 *B*).

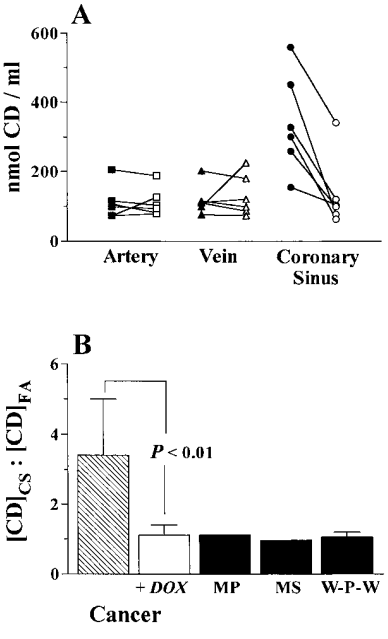

Figure 2. Effects of 60 mg DOX/m² on the circulating levels of CD in cancer patients. (*A*) CD were measured in the coronary sinus, femoral artery and antecubital veins of six cancer patients, (# 1–6 in Table I). Blood was collected before (*solid symbols*) and 5 min after a bolus of 60 mg DOX/m² i.v. (*open symbols*), as described in Methods. (*B*) The results are expressed as changes of $[CD]_{CS}/[CD]_{FA}$ ratio and compared with base-line values in patients suffering from Wolff-Parkinson-White disease (*W-P-W*) and mitral valve prolapse (*MP*) or stenosis (*MS*), respectively. See also text for explanations.

Table III. Dose-dependent Effects of DOX in Patients with Different $[CD]_{CS}/[CD]_{FA}$ Ratios

		nmol CD/ml		
Patient*	mg DOX/m²	Coronary sinus	Femoral artery	$[CD]_{CS}/[CD]_{FA}$
7‡	—	107.9	94.2	1.1
	25	106.9	84.1	1.3
8§	—	573.5	147.3	3.9
	13	143.3	133.5	1.1
	47	121.7	130.4	0.9
9§	—	275.9	304.4	0.9
	13	196.6	255.4	0.8
	47	221.8	199.6	1.1

All blood samples were splitted, processed for plasma separation and analyzed in duplicate, with interassay agreement ≥ 90%. Values are means of the duplicate measurements. *Compare with Table I for clinical data. ‡CD were measured before and 5 min after a single infusion of 25 mg DOX/m². §These patients were given two consecutive infusions with an interdose interval of 10 min. CD were measured before treatment and five min after each infusion.

Dose-dependent effects of DOX in patients with different $[CD]_{CS}/[CD]_{FA}$ ratios. Cardiac lipid peroxidation was studied in three patients undergoing modified DOX treatments. One of these patients had been previously treated with irradiation of mediastinum; hence, he had both clinical and ethical indications for dose restriction and was scheduled to receive infusions of 25 mg/m² in place of the usual 60 mg/m² regimen (Methods and Table I). In the other patients, the standard dose of 60 mg/m² was fractionated in two consecutive 10-min–interval infusions of 13 and 47 mg/m², to evaluate the dose-dependence of DOX effects. As shown in Table III, these three patients exhibited major differences in the basal levels of CD in femoral artery and coronary sinus. Upon appropriate calculations, it turned out that the patient scheduled to receive a single infusion of 25 mg/m² had a $[CD]_{CS}/[CD]_{FA}$ ratio of 1.1, whereas the two patients scheduled to receive consecutive infusions of 13 and 47 mg/m² had $[CD]_{CS}/[CD]_{FA}$ ratios of 0.9 or 3.9. In patients with $[CD]_{CS}/[CD]_{FA}$ ratios around unity, one single infusion of 25 mg/m² or two consecutive infusions of 13 and 47 mg DOX/m² had relatively marginal and simultaneous effects on the CD levels in coronary sinus and femoral artery, leaving the corresponding $[CD]_{CS}/[CD]_{FA}$ ratios virtually unaltered (Table III). Thus, DOX failed to stimulate lipid peroxidation in cardiac tissues that were not spontaneously involved in this process. Table III also shows that the patient with a $[CD]_{CS}/[CD]_{FA}$ ratio of 3.9 responded to the infusion of 13 mg DOX/m² with a 75% decrease of CD in coronary sinus vs a 9% decrease in femoral artery; therefore, the $[CD]_{CS}/[CD]_{FA}$ ratio decreased to 1.1. Subsequent infusion of 47 mg DOX/m² had minor effects on the CD levels in femoral artery or coronary sinus, leaving the $[CD]_{CS}/[CD]_{FA}$ ratio around unity (Table III). In this patient, cardiac lipid peroxidation was also studied by measuring LOOH. Before treatment, LOOH were exceedingly higher in coronary sinus than in femoral artery; however, this difference was nearly abolished by the infusion of 13 mg DOX/m², which decreased LOOH in coronary sinus but not in

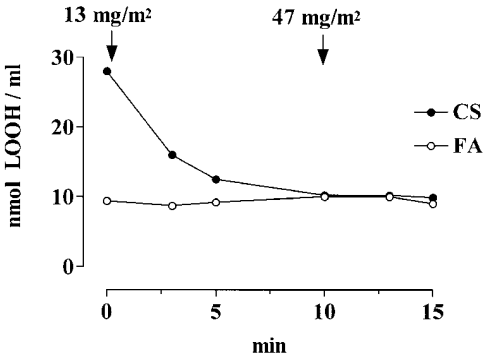

Figure 3. Effects of two consecutive DOX infusions on LOOH levels in coronary sinus and femoral artery. LOOH were measured in blood samples from the coronary sinus and femoral artery of patient 8, before and after consecutive infusions of 13 and 47 mg DOX/m^2. All conditions were as described in Methods or Table III, with the exception that LOOH were also measured in additional samples collected 3 min after each infusion.

femoral artery (Fig. 3). Subsequent infusion of 47 mg DOX/m^2 had minor or no effect on the LOOH levels in either vascular site (see also Fig. 3). Thus, LOOH measurements were in excellent agreement with CD determinations, showing that as little as 13 mg DOX/m^2 could potently inhibit lipid peroxidation in cardiac tissues where this process was spontaneously enhanced.

ADP-Fe(II) as a possible catalyst for cardiac lipid peroxidation. Experiments were performed in vitro to characterize iron source(s) possibly involved in the spontaneous and DOX-inhibitable cardiac lipid peroxidation. For this purpose, myoglobin was reconstituted with H_2O_2 and arachidonic acid, i.e., a major product of the redox cycling of DOX and a predominant substrate of membrane lipid peroxidation (48). As shown in Table IV, neither myoglobin nor H_2O_2 could promote lipid peroxidation. Extensive formation of CD occurred upon coincubation of myoglobin with H_2O_2, but LOOH were not formed under these conditions (Table IV). Lipid peroxidation was further investigated by replacing myoglobin with ADP-Fe(II), reportedly a chelate of pathophysiologic relevance (34). As also shown in Table IV, ADP-Fe(II) was found to generate both CD and LOOH by virtue of a direct mechanism that did not require H_2O_2. This effect was unambiguously mediated by a chelator–iron complex, as neither Fe(II) nor ADP yielded LOOH or CD when incubated separately with arachidonic acid. Importantly, the addition of H_2O_2 did not increase, but actually inhibited ADP/Fe(II)-dependent lipid peroxidation, as reflected by the simultaneous decrease of CD and LOOH (Table IV).

Lipid peroxidation was further investigated by measuring TBARS. This assay has its inherent limitations for in vivo studies (21, 28); however, it is suitable for in vitro experiments and measures a variety of compounds, involving byproducts of LOOH decomposition as well as endoperoxides that generate malondialdehyde (24, 35, 49). As shown in Fig. 4, myoglobin effectively converted arachidonic acid into TBARS, provided that H_2O_2 was included to achieve increasing ratios with the heme iron moiety. This finding showed that myoglobin was an effective catalyst of lipid peroxidation, as it could generate CD that converted into oxygenated products with TBA reactivity. In this setting, lack of LOOH detection by the xylenol orange/Fe(II) assay probably reflected that myoglobin decomposed

Table IV. Myoglobin vs ADP/Fe(II)-dependent Lipid Peroxidation

System	CD	LOOH
	nmol/ml	
Mb	ND	ND
H_2O_2	ND	ND
Mb + H_2O_2	18.8±2	ND
ADP	ND	ND
Fe(II)	ND	ND
ADP-Fe(II)	19.7±2.4	7.9±0.3
ADP-Fe(II) + H_2O_2	8.1±0.7*	1.7±0.8*

Incubations (0.5–1 ml final volume) contained arachidonic acid vesicles (0.4 mM) in 0.3 M NaCl, pH 7.0, 37°C. Myoglobin-dependent systems included the heme protein (0.2 mM) and/or H_2O_2 (4 mM). ADP/iron-dependent systems contained $FeSO_4$ (0.1 mM) and/or the chelator (1 mM). Where indicated, H_2O_2 (2 mM) was also included to achieve the same 20:1 ratio with iron as obtained in the experiments with myoglobin. After a 30-min incubation, catalase (400 U/ml) was included to decompose unreacted H_2O_2, and lipid peroxidation was measured by assaying for CD and LOOH, as described in Methods. Values are means±SD of 3–4 separate determinations.*Significantly different from incubations lacking H_2O_2 ($P < 0.01$). Mb, metmyoglobin; ND, not detectable.

the hydroperoxide moieties too rapidly for them to be measured, or it favored peroxidation pathways involving endoperoxides in place of LOOH. At the same time, these results ruled out the involvement of myoglobin in DOX-inhibitable cardiac lipid peroxidation. In fact, the formation of CD and TBARS required increasing amounts of H_2O_2; hence, this was not the mechanism whereby lipid peroxidation ceased when DOX reduced oxygen to H_2O_2. Fig. 4 also shows that ADP-Fe(II) per se was capable of forming TBARS from arachidonic acid; however, the reaction was inhibited by H_2O_2 and such inhibition became increasingly evident as the H_2O_2/Fe(II) ratio exceeded unity (Fig. 4). It follows that spontaneous and DOX-inhibitable cardiac lipid peroxidation probably involved ADP-Fe(II), as the ability of this complex to generate CD, LOOH, and TBARS was uniformly suppressed when H_2O_2 was included to simulate DOX infusion and redox cycling. Histidine-Fe(II), another complex of pathophysiologic relevance, was similarly tested for its ability to promote lipid peroxidation. Neither CD nor LOOH or TBARS were significantly formed

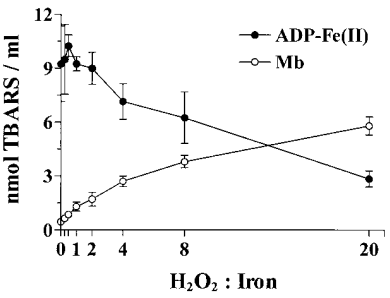

Figure 4. Myoglobin- vs ADP/Fe(II)-dependent lipid peroxidation. Incubations (0.5 ml final volume) contained arachidonic acid vesicles (0.4 mM) and either metmyoglobin (0.2 mM) or ADP-Fe(II) (1 mM chelator–0.1 mM $FeSO_4$) in 0.3 M NaCl, pH 7.0, 37°C. Increasing amounts of H_2O_2 were also included to achieve molar ratios with iron, as indicated. After 30 min, catalase (400 U/ml) was included to decompose unreacted H_2O_2 and samples were assayed for TBARS as described in Methods. Values are means±SD of 3–5 determinations.

Table V. Nonheme Nonferritin Iron in Native or ADP/Fe(II)-supplemented Cytosolic Fractions from Human Liver or Heart

Tissue	Fe(II)	Fe(III)
	nmol iron/8 mg protein	
Heart		
Native	2.5	1.8
+ ADP-Fe(II)	2.6	1.7
Liver		
Native	1.5	1.6
+ ADP-Fe(II)	1.4	1.7

Cytosolic fractions from human heart or liver biopsies (8 mg protein/ml) were gel filtered on Sepharose 6B before and after a 30-min incubation at 37°C with ADP-Fe(II) (1 mM chelator–0.1 mM $FeSO_4$). Nonheme nonferritin iron was identified and measured in chromatographed fractions as described in Methods. Values are taken from a representative experiment.

Table VI. Ferritin Iron in Native or ADP/Fe(II)-supplemented Cytosolic Fractions from Human Liver or Heart

Tissue	Fe(II)	Fe(III)	Net iron incorporation [Fe(II) + Fe(III)]
	nmol iron/8 mg protein		
Heart			
Native	10.4	15.2	
+ ADP-Fe(II)	8.8	60.0	43.2
Liver			
Native	4.0	38.4	
+ ADP-Fe(II)	12.8	121.6	92.0

All experimental conditions were as described in Table V, except that chromatographed fractions were assayed for ferritin iron. Values are taken from a representative experiment.

by this complex within the same H_2O_2/iron ratios used in myoglobin- or ADP/Fe(II)-dependent systems (not shown).

Role of ferritin and nonferritin proteins in cardiac iron sequestration and lipid peroxidation. Having implicated ADP-Fe(II) as a possible catalyst of myocardial lipid peroxidation, we thought it important to evaluate how this complex interacted with iron-storage proteins. As shown in Table V, cytosols contained Fe(II) and Fe(III) ions that were bound to protein(s) other than ferritin, in agreement with an earlier proposal for the existence of nonheme nonferritin iron stores (41). Preincubation of cytosols with ADP-Fe(II) did not modify the size or redox state of nonferritin pools of iron, indicating that the binding protein(s) could not exchange iron with this particular chelate. Similar results were obtained with cytosols from extracardiac tissues, e.g., liver (Table V). The vast majority of cytosolic iron was bound to ferritin. In hepatic samples, greater than 90% of ferritin-bound iron was recovered in a ferric form, whereas in cardiac samples iron ions were almost equally distributed in ferrous and ferric forms (Table VI). This showed that functional differences may exist between cardiac and hepatic isoferritins with respect to the mechanisms of iron incorporation and redox stabilization. Therefore, ferritin-bound Fe(II) and Fe(III) ions were also measured in cytosols that had been incubated with ADP-Fe(II) to simulate a pathologic increase in the availability of this complex. Both cardiac and hepatic cytosols responded to ADP-Fe(II) supplementation by incorporating sizable amounts of iron into ferritin. In particular, we found that: (*a*) 43 or 92% of iron was partitioned from ADP-Fe(II) and deposited in myocardial or liver isoferritins, respectively; and (*b*) greater than 90% of iron was incorporated in a ferric form (Table VI). In the absence of cytosols, only 25.2% of ADP-chelated Fe(II) oxidized with oxygen to form Fe(III); therefore, the results with ADP-Fe(II) plus cytosols showed that: (*a*) cardiac and extracardiac tissues shared a common mechanism, involving the oxidation of Fe(II) and the incorporation of Fe(III) in ferritin; and (*b*) the overall process of iron sequestration was less effective in cardiac cytosols than in liver samples. Keeping this difference in mind, cytosolic fractions from myocardial or liver biopsies were tested for their ability to prevent arachidonic acid peroxidation by ADP-Fe(II). As shown in Fig. 5, cardiac cytosols inhibited lipid peroxidation less efficiently than did hepatic samples, with an apparent IC_{50} of 2.2 vs 1.2 mg protein/ml, respectively. After ferritin removal by immunoaffinity chromatography, both tissue samples lost their ability to suppress lipid peroxidation, providing direct evidence that oxidant damage was inhibited by the iron-sequestering activity of this storage protein (Fig. 5). Collectively, the experiments in Table VI and Fig. 5 showed that ferritin content and/or functions limited the ability of cardiac tissues to sequester iron and prevent lipid peroxidation. Cytosolic fractions from myocardial biopsies did contain less ferritin than liver samples (Fig. 6); however, a 29% decrease in ferritin protein could not account for a 53% decrease in iron incorporation (Table VI) or 83% increase in the IC_{50} for lipid peroxidation (Fig. 5), suggesting that the sequestration of iron and the inhibition of lipid peroxidation were influenced by additional factor(s). In this respect, SDS-PAGE showed that liver ferritin was composed predominantly of $M_r \sim 20{,}000$ light (L) subunits, whereas heart ferritin contained a larger quantity of $M_r \sim 23{,}000$ heavy (H) subunits (Fig. 6, right). Considering that subunit composition modulates ferritin functions (50), we hypothesized that differences in H:L ratios were important and determined tissue-specific

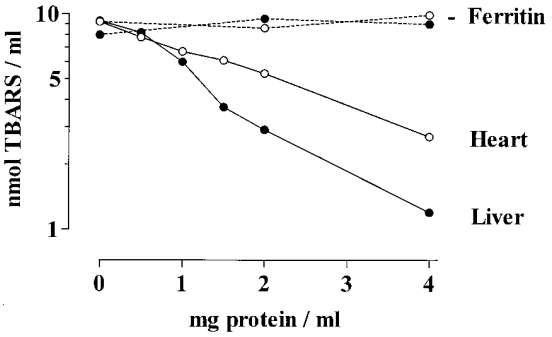

Figure 5. Inhibition of ADP/Fe(II)-dependent lipid peroxidation by cardiac or hepatic cytosols. Incubations (1 ml final volume) contained arachidonic acid vesicles (0.4 mM), ADP-Fe(II) (1 mM chelator–0.1 mM $FeSO_4$) and increasing amounts of cytosolic fractions from hepatic or cardiac biopsies in 0.3 M NaCl, pH 7.0, 37°C. After 30 min, the incubations were assayed for lipid peroxidation by measuring TBARS. Where indicated, cytosols had been previously subjected to immunoaffinity chromatography to remove ferritin, as described in Methods. Values are means of two separate determinations with 80% experimental agreement.

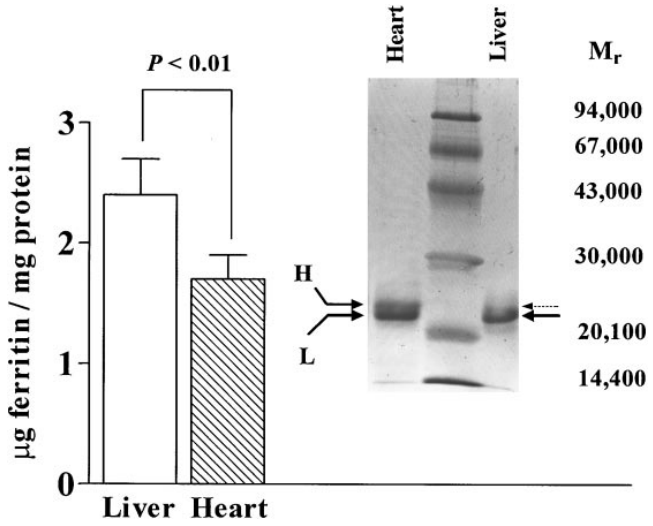

Figure 6. Ferritin content and subunit composition in cardiac or hepatic cytosols. Ferritin was measured by titration with bicinchoninic acid in Mg eluants from CNBr-Sepharose 2B immunoaffinity columns, as described in Methods. Values are means±SD of three separate determinations. The right panel shows an electrophoretogram (SDS-PAGE) where left and right lanes were loaded with ~ 5 μg of immuno-chromatographed heart or liver ferritin, respectively. The central lane 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.

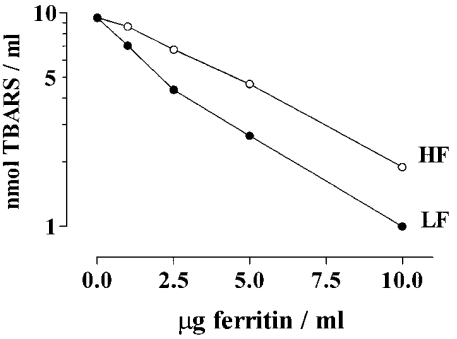

Figure 7. Inhibition of ADP/Fe(II)-dependent lipid peroxidation by liver and heart ferritin. All experimental conditions were as described in Fig. 5, with the exception that cytosols were replaced with ferritins prepared by immunoaffinity chromatography, as described in Methods. Values are taken from a representative experiment. HF, heart ferritin; LF, liver ferritin.

responses to the availability of ADP-Fe(II). This hypothesis was tested by reconstituting arachidonic acid and ADP-Fe(II) with heart or liver ferritins that had been purified by immunoaffinity chromatography of the corresponding cytosolic fractions. As shown in Fig. 7, heart ferritin was less effective than liver ferritin in inhibiting lipid peroxidation, with an apparent IC_{50} of 4.4 vs 2.1 μg, respectively. Thus, ferritin subunit composition limited the ability of cardiac tissues to partition iron from ADP before it catalyzed lipid peroxidation, possibly explaining how cancer patients often exhibited a spontaneous release of CD and LOOH in the coronary sinus.

In a final set of experiments, we addressed the hypothesis that DOX treatment and H_2O_2 formation might have inhibited cardiac lipid peroxidation by increasing the oxidation of Fe(II), which is coupled with the incorporation of Fe(III). Therefore, ADP-Fe(II) oxidation and Fe(III) incorporation were studied in native or H_2O_2-supplemented cardiac cytosols. In native cytosols, ADP-Fe(II) oxidation was rather tightly coupled with the incorporation of Fe(III) in ferritin, as evidenced by a stoichiometry of Fe(II) oxidation vs Fe(III) incorporation of 1.4 (Table VII). These experiments were repeated after the addition of H_2O_2, yielding the 20:1 ratio to Fe(II), which had previously been shown to suppress lipid peroxidation (Table IV and Fig. 4). Under these conditions, H_2O_2 did increase the oxidation of Fe(II) but simultaneously abolished the incorporation of Fe(III), raising the stoichiometry of Fe(II) oxidation vs Fe(III) incorporation from 1.4 to 14.9 (Table VII). These results showed that ferritin could not acquire Fe(III) from the reaction of Fe(II) with H_2O_2. Therefore, DOX treatments and H_2O_2 formation inhibited cardiac lipid peroxidation not by decreasing the availability of iron, but rather by affecting the mechanism(s) whereby ADP-Fe(II) reacted with lipids (Table IV and Fig. 4). Importantly, immunoaffinity chromatography and ferritin removal made cytosols ineffective not only with respect to the incorporation of Fe(III), but also with respect to the oxidation of Fe(II) (Table VII). This showed that the "ferroxidase" activity of cytosols was entirely contingent on the availability of ferritin.

Discussion

Membrane lipid peroxidation proceeds via hydrogen abstraction from a *bis*-allylic bond of polyunsaturated phospholipids, yielding an alkyl radical and causing rearrangement of double bonds in the form typical of a conjugated diene. Subsequent

Table VII. Effects of H_2O_2 Addition or Ferritin Removal on ADP-Fe(II) Oxidation and Fe(III) Incorporation in Cardiac Cytosols

			Fe(II) oxidation*
System	Fe(II) oxidation	Fe(III) incorporation	Fe(III) incorporation
	nmol/8 mg protein		
ADP-Fe(II)	25.2		
+ cytosol	84.0	42	1.4
+ cytosol + H_2O_2	100	5	14.9
+ cytosol − ferritin	27.2	ND	—

Cytosolic fractions from myocardial biopsies (8 mg protein/ml) were incubated with ADP-Fe(II) and assayed for Fe(II) oxidation and Fe(III) incorporation in ferritin fractions, as described in Methods. In the experiments with H_2O_2 (2 mM), cytosols were first treated with sodium azide (0.1 mM) to inhibit trace catalase, and then dialyzed against 0.3 M NaCl just before use. Where indicated, cytosols had been subjected to immunoaffinity chromatography to remove ferritin. Values are taken from representative experiments.

*For the calculation of this ratio, net values of Fe(II) oxidation were obtained by correction for the spontaneous loss of bathophenanthroline-chelatable Fe(II) in cytosol-free incubations, or for the increase in ferritin-bound Fe(II) at the end of incubation (Table VI). ND, not detectable.

reaction of alkyl radicals with molecular oxygen generates peroxyl radicals that abstract hydrogen from a neighboring allylic bond, yielding LOOH and new CD. Once formed in membranes, free radical–oxidized lipids can be released into extracellular fluids, presumably by the action of phospholipase A_2 (51, 52). In keeping with this general mechanism, the circulating levels of CD and LOOH have been found to increase under clinical conditions characterized by free radical formation and membrane lipid peroxidation (53–55). With regard to cancer patients, there have been reports of a possible increase of plasma TBARS (56); however, this phenomenon does not always approach statistical significance (57). While the methodologic aspects and pathophysiologic significance of plasma TBARS remain a matter of controversy (21, 28, 56, 57), studies with nuclear magnetic resonance techniques have confirmed that human malignancies often accompany with high plasma levels of oxidized lipids, which have been interpreted as biochemical evidence for LDL damage (58). Our present studies modify and extend those reports, showing that cancer patients have circulating levels of CD and LOOH that reflect membrane lipid peroxidation rather than LDL oxidation or free radical–independent modifications of dietary lipids (Results, Table II, and Fig. 1, *A–C*). Cancer-associated nutritional disturbances (59) and deficiencies in α-tocopherol, selenium and other antioxidant factors (60, 61) probably contribute to the maintenance of one such condition of systemic lipid peroxidation. In our study, CD and LOOH have also been measured, for the first time, in blood plasma samples collected from the coronary sinus of cancer patients before and after intravenous treatments with DOX, a chemotherapeutic agent that is thought to stimulate cardiac lipid peroxidation by overwhelming the local defences against oxygen free radicals. These measurements have shown that: (*a*) cancer patients may have a spontaneous exacerbation of cardiac lipid peroxidation, as evidenced by the increase of CD and LOOH in coronary sinus vs femoral artery or antecubital veins; and (*b*) cardiac lipid peroxidation is inhibited by DOX infusion, with such inhibition becoming evident in response to as little as 13 mg DOX/m^2, i.e., less than one fourth of the dose usually recommended for single infusions (Table III and Figs. 2 and 3). In principle, drugs affecting the circulating levels of CD and LOOH may act by protecting membranes from lipid oxidants and/or by interfering with phospholipases. In this respect, previous studies in laboratory animals have shown that DOX does not inhibit myocardial phospholipases A_1 or A_2, nor does it affect lysophospholipases or acylCoA: lysophosphatidil choline acyltransferases that participate in the physiologic turnover of membrane lipids (62). In vitro, DOX would actually stimulate phospholipase A_2 (63). Keeping these premises in mind, we cannot escape the conclusion that DOX may decrease the cardiac release of CD and LOOH by "protecting" membranes from free radical reactions, thus behaving as a sort of antioxidant rather than an oxidant. Myocardium sampling and in situ measurements of CD or LOOH would be much needed to corroborate this conclusion and rule out the possibility that an oxidant burden has occurred within inaccessible intracellular environments. While such invasive measurements are precluded by ethical and practical constraints, our indirect determinations in coronary sinus provide a rationale to discuss alternative mechanism(s) of anthracycline cardiotoxicity.

Studies in vitro with various sources of heme and nonheme iron have led us to implicate ADP-Fe(II) as a possible catalyst of spontaneous and DOX-inhibitable cardiac lipid peroxidation, mostly because the ability of this complex to generate CD, LOOH, and TBARS decreases in the presence of H_2O_2, i.e., under conditions resembling the redox cycling of DOX and the accumulation of H_2O_2 in catalase- and glutathione peroxidase-deficient cardiomyocytes (Fig. 4 and Table IV). These observations indicate that cardiac lipid peroxidation proceeds via reactive intermediates that must be different from hydroxyl radical, as the formation of this species would be favored, rather than inhibited, by H_2O_2 (8). In this setting, Minotti and Aust (32, 33, 64) and several other research groups (65–70) have proposed that the hydroxyl radical cannot promote lipid peroxidation, inasmuch as it exhibits a diffusion-limited reactivity and cannot migrate from the site(s) of generation to the hydrophobic membrane phases where the *bis*-allylic bonds are buried. Lipid peroxidation would occur anytime iron oxidizes incompletely to the ferric form, yielding perferryl ions [$Fe(II)O_2$–$Fe(III)O_2^-$] or oxygen-bridged Fe(II)–Fe(III) complexes that are more stable and can substitute for hydroxyl radical (33, 64–67). This process is favored when iron is chelated by ADP and oxygen serves as the oxidant (31, 32, 67). Once initiated by iron-oxygen complexes, lipid peroxidation "propagates" through the decomposition of LOOH to highly reactive alkoxyl radicals which reinitiate hydrogen abstraction (8). The formation of Fe(II):Fe(III) ratios is important also for propagation; in fact, some Fe(II) is needed for the decomposition of LOOH, but excess Fe(II) or Fe(III) would terminate propagation by reducing alkoxyl radicals to hydroxy compounds or by converting LOOH into unreactive carbonyls, respectively (8). One obvious implication of these mechanisms is that H_2O_2 can paradoxically inhibit lipid peroxidation by oxidizing too much Fe(II) to Fe(III), thus precluding initiation and propagation reactions that involve Fe(II)⇔Fe(III) equilibrium (8, 33, 64, 65). Viewed in this context, our in vitro and in vivo studies would therefore suggest that: (*a*) cardiomyocytes have endogenous levels of ADP-Fe(II), which promotes lipid peroxidation by oxidizing incompletely with oxygen; and (*b*) lipid peroxidation ceases at the time when DOX is infused and the excessive formation of H_2O_2 drives a complete oxidation of iron from Fe(II) to Fe(III). According to previous studies, Fe(II)⇔Fe(III) equilibrium and lipid peroxidation might also be affected by the formation of a DOX-Fe(II) complex that is highly unstable and tends to oxidize completely with oxygen (3, 8, 71). This may not occur with preexisting iron complexes; in fact, control experiments showed that DOX could not compete with ADP for Fe(II), nor did it form spectrally or chromatographically evident iron complexes under these conditions (not shown). Thus, semiquinone formation and H_2O_2 formation emerge as the predominant factors inhibiting cardiac lipid peroxidation by preformed ADP-iron complexes.

Spontaneous exacerbation of myocardial lipid peroxidation cannot be seen in cancer-free patients undergoing cardiac catheterization for the evaluation of valvular or arrhythmogenic diseases (Fig 2 *B*). Thus, it is the presence of cancer that influences cardiac lipid peroxidation, perhaps by favoring the formation of an intracellular pool of low mol wt iron. In this respect, studies in tumor-bearing rodents have shown that cancer growth may accompany with systemic induction of heme oxygenase, the enzyme that cleaves the porphyrin ring of heme proteins and liberates iron that is coordinated therein (72). In principle, cancer development and host-defense reactions might also result in the formation of cytokines, such as in-

terleukin-2, that selectively upregulate the synthesis of transferrin receptor, but not of ferritin, thus creating a potential imbalance between cellular iron uptake and sequestration (73). These limited examples suggest that cancer growth may be linked to systemic processes increasing the formation of an intracellular pool of low mol wt iron. Irrespective of the precise mechanism(s) affecting iron homeostasis, more severe consequences should be anticipated in cardiomyocytes than in other cell types. In fact, ferritin sequesters iron more efficiently when the oxidation of Fe(II) is catalyzed by a limited number of H subunits and the accommodation of Fe(III) is made sterically favorable by a larger number of L subunits (50). This is not the case for cardiac ferritin, having more H than L subunits as compared with extracardiac samples; e.g., liver isoferritins (Fig. 6 and reference 44). Accordingly, comparative experiments have shown that cytosolic fractions or purified ferritins from cardiac tissues are less effective than liver samples in sequestering iron from ADP and preventing lipid peroxidation (Table VI and Figs. 5 and 7), possibly explaining how the peroxidative tone of cancer patients is selectively enhanced at the myocardial level. From a mechanistic viewpoint, previous studies have raised the possibility that iron homeostasis may also be governed by protein(s) and function(s) other than ferritin and its ferroxidase activity. In particular, some research groups have characterized the existence of "nonferritin" storage proteins (74, 75), whereas others have proposed that ferritin would acquire Fe(III) through the ferroxidase activity of ceruloplasmin (76). We have addressed these possibilities, but our data indicate that ADP-Fe(II) behaves as a rather unique iron complex, in the sense that it cannot be oxidized or incorporated by proteins other than ferritin (Tables V and VII). Having shown this, we have also been able to demonstrate that H_2O_2 does not assist the oxidative incorporation of iron and actually uncouples the formation of Fe(III) from its deposition in ferritin (Table VII). These observations lend support to our proposal that DOX treatments and H_2O_2 formation would inhibit cardiac lipid peroxidation by solely affecting the Fe(II)⇔Fe(III) equilibrium of iron–oxygen complexes. While inhibiting cardiac lipid peroxidation in patients with $[CD]_{CS}/[CD]_{FA}$ ratios > 1, DOX may also fail to promote lipid damage in those few subjects with $[CD]_{CS}/[CD]_{FA}$ ratios ~ 1 (Table III). This implies that DOX cannot promote the peroxidation of cardiac tissues in which iron homeostasis is most probably conserved. Again, the involvement of lipid oxidants other than hydroxyl radical may shed light on these otherwise unexplainable findings. In fact, we have previously shown in vitro that DOX metabolism and semiquinone formation reductively release iron from ferritin or other newly identified microsomal proteins; howewer, lipid peroxidation is precluded by simultaneous formation of a stoichiometric excess of H_2O_2, causing the oxidation of too much Fe(II) to Fe(III) (71, 77). This dual process of iron delocalization vs redox-inactivation may very well occur in cardiac tissues with impaired H_2O_2 disposition, explaining the ineffectiveness of DOX infusions in patients with base-line $[CD]_{CS}/[CD]_{FA}$ ratios ~ 1.

Previous in vitro evidence for the involvement of iron and free radicals in DOX toxicity has provided a rationale for pharmacologic interventions with antioxidants or chelators. Unfortunately, the results of clinical studies have remained a matter of controversy. In a limited trial, the progressive decline of cardiac functions was not prevented by supplementing patients with a major lipid-soluble antioxidant such as α-tocopherol (78). Likewise, no significant cardioprotection was observed in a randomized controlled trial assessing the effects of *N*-acetylcisteine, which contributes to the antioxidant repertoire of the cell by maintaining adequate levels of reduced glutathione (79). Our present studies clarify the molecular basis of these negative reports, showing that DOX fails to aggravate cardiac lipid peroxidation and probably inhibits it in a paradoxic manner. It follows that cardiac damage might not be caused by lipid peroxidation, nor should antioxidants be expected to afford protection. Cardioprotection has nevertheless been observed with dexrazoxane, a bispiperazinedione that hydrolyzes intracellularly and liberates a diacid diamide that chelates iron (80). These findings have provided evidence for the involvement of iron in the clinical manifestations of cardiac damage. However, recent in vitro studies have also shown that a complex of iron with the diacid diamide generates hydroxyl radical (81), thus implying that cardioprotection is achieved in the face of a persistent generation of this oxidant. In the present study, we did not address the molecular mechanism(s) whereby iron chelation would mitigate cardiac damage. Our arguments against the involvement of hydroxyl radical in oxidative injury would nevertheless explain how dexrazoxane can afford protection in spite of the redox activity of diacid diamide–iron complexes.

While providing a mechanism to reconcile previous diverging evidence, our results set the stage to direct future research and therapeutic interventions toward molecular target(s) possibly different from lipid peroxidation. Thus, cardiac damage might very well originate from free radical–independent processes that involve unmetabolized DOX or drug metabolites other than semiquinones. The latter possibility should not be neglected, as we have recently shown in vitro that secondary alcohol metabolites of the side chain of DOX delocalize nonferritin sources of iron via reaction mechanisms preluding to the dysfunction of iron-requiring enzymes, rather than to the initiation of iron-catalyzed free radical damage (41). This and other mechanisms of toxicity will have to be validated directly in cancer patients rather than in animal models, provided that ethically acceptable and well-tolerable procedures are designed and respected. In fact, our observations on the different nature and levels of CD in cancer patients vs healthy subjects, or other diseased individuals, suggest that neoplasia causes or accompanies with systemic and heart-specific modifications that can be important in determining the individual response to DOX treatments. The entire spectrum of cancer-related modifications is probably impossible to reproduce in any given model of tumor-transplanted animal. In fact, the quality and intensity of metabolic or immunologic adaptations to cancer development may unpredictably depend on the animal species and strain. It should also be noted that studies in small-size tumor-bearing rodents often require that DOX be given intraperitoneally (13, 14), thus creating a "first-pass" hepatic metabolism that alters drug availability and generates additional differences from clinical conditions of intravenous treatments. Studies in cancer patients will therefore remain crucial to elucidate the molecular mechanism(s) of cardiotoxicity and characterize the action of dexrazoxane or other cardioprotectants.

References

1. Minow, R.A., R.S. Benjamin, and J.A. Gottlieb. 1975. Adriamycin (NSC 123127) cardiomyopathy—an overview with determination of risk factors. *Can-*

cer Chemother. Rep. 6:198–201.

2. Watts, R.G. 1991. Severe and fatal anthracycline cardiotoxicity at cumulative doses below 400 mg/m^2: evidence for enhanced toxicity with multiagent chemotherapy. *Am. J. Hematol.* 36:217–218.

3. Powis, G. 1989. Free radical formation by antitumor quinones. *Free Radic. Biol. & Med.* 6:63–101.

4. Olson, R.D., and P.S. Mushlin. 1990. Doxorubicin cardiotoxicity: analysis of prevailing hypotheses. *FASEB J.* 4:3076–3086.

5. Doroshow, J.H. 1991. Doxorubicin-induced cardiotoxicity. *N. Engl. J. Med.* 324:843–845.

6. Borrello, S., E. De Leo, H. Wohlrab, and T. Galeotti. 1992. Manganese deficiency and transcriptional regulation of mitochondrial superoxide dismutase. *FEBS Lett.* 3:249–254.

7. Mordente, A., S.A. Santini, G.A.D. Miggiano, G.E. Martorana, T. Petitti, G. Minotti, and B. Giardina. 1994. The interaction of short-chain coenzyme Q analogs with different redox state of myoglobin. *J. Biol. Chem.* 269:27394–27400.

8. Minotti, G. 1993. Sources and role of iron in lipid peroxidation. *Chem. Res. Toxicol.* 6:134–146.

9. Theil, E.C. 1983. Ferritin: structure, function, and regulation. *In* Iron Binding Proteins Without Cofactors or Sulfur Clusters. E.C. Theil, G.L. Eichorn, and L. Marzili, editors. Elsevier Science Publishing, Co., Inc., New York. 1–38.

10. Ryan, T.P., and S.D. Aust. 1992. The role of iron in oxygen-mediated toxicities. *Crit. Rev. Toxicol.* 22:119–141.

11. Rajagopalan, S., P.M. Politi, B.K. Sinha, and C.E. Myers. 1988. Adriamycin-induced free radical formation in the perfused rat heart: implications for cardiotoxicity. *Cancer Res.* 48:4766–4769.

12. Tesoriere, L., M. Ciaccio, M. Valenza, A. Bongiorno, E. Maresi, R. Albiero, and M.A. Livrea. 1994. Effect of vitamin A administration on resistance of rat heart against doxorubicin-induced cardiotoxicity and lethality. *J. Pharmacol. Exp. Ther.* 269:430–436.

13. Siveski-Iliskovic, N., M. Hill, D.A. Chow, and P.K. Singal. 1995. Probucol protects against adriamycin cardiomyopathy without interfering with its antitumor effect. *Circulation.* 91:10–15.

14. Sridhar, R., C. Dwivedi, J. Anderson, P.B. Baker, H.M. Sharma, P. Desai, F.N. Engineer. 1992. Effects of verapamil on the acute toxicity of doxorubicin in vivo. *J. Natl. Cancer Inst.* 84:1653–1659.

15. Fischer, J.C., R.L. Tackett, E.W. Howerth, and M.A. Johnson. 1992. Copper and selenium deficiencies do not enhance the cardiotoxicity in rats due to chronic doxorubicin treatment. *J. Nutr.* 122:2128–2137.

16. Baird, M.B., J.L. Hough, and G.T. Sfeir. 1993. Accumulation of malondialdehyde in mouse heart following acute dosing with adriamycin is strain specific and unaffected by cardiac catalase status. *Res. Commun. Chem. Pathol. Pharmacol.* 80:363–366.

17. Praet, M., and J.M. Ruysschaert. 1993. In vivo and in vitro mitochondrial membrane damages induced in mice by adriamycin and derivatives. *Biochim. Biophys. Acta.* 1149:79–85.

18. Mor, V., L. Laliberte, J.N. Morris, and M. Wiemann. 1984. The Karnofsky performance status scale: an examination of its reliability and validity in a research setting. *Cancer.* 53:2002–2007.

19. Ledwozyw, A., J. Michalak, A. Stepien, and A. Kadziolka. 1986. The relationship between plasma triglycerides, cholesterol, total lipids and lipid peroxidation products during human atherosclerosis. *Clin. Chim. Acta.* 155:275–284.

20. Brown, K.M., P.C. Morrice, and G. Duthie. 1994. Vitamin E supplementation suppresses indexes of lipid peroxidation and platelet counts in blood of smokers and nonsmokers but plasma lipoprotein concentrations remain unchanged. *Am. J. Clin. Nutr.* 60:383–387.

21. Morrow, J.D., B. Frei, A.W. Longmire, J.M. Graziano, S.M. Lynch, Y. Shyr, W.E. Strauss, J.A. Oates, and L.J. Roberts. 1995. Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. *N. Engl. J. Med.* 332:1198–1203.

22. Seldinger, S. 1953. Catheter replacement of the needle in percutaneous arteriography: a new technique. *Acta Radiol.* 39:368–376.

23. Folch, J.M., M. Lees, and H.S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497–509.

24. Ambrosio, G., J.T. Flaherty, C. Duilio, I. Tritto, G. Santoro, P.P. Elia, M. Condorelli, and M. Chiariello. 1991. Oxygen radical generated at reflow induce peroxidation of membrane lipids in reperfused hearts. *J. Clin. Invest.* 87: 2056–2066.

25. Corongiu, F.P., S. Banni, and M.A. Dessi'. 1989. Conjugated dienes detected in tissue lipid extracts by second-derivative spectrophotometry. *Free Radical Biol. & Med.* 7:183–186.

26. Situnayake, R.D., B.J. Crump, A.V. Zezulka, M. Davis, B. McConkey, and D.I. Thurnham. 1990. Measurement of conjugated diene lipids by derivative spectroscopy in heptane extracts of plasma. *Ann. Clin. Biochem.* 27:258–266.

27. Jiang, Z.Y., J.V. Hunt, and S.P. Wolff. 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxides in low density lipoprotein. *Anal. Biochem.* 26:853–856.

28. Nourooz-Zadeh, J., J. Tajaddini-Sarmadi, and S.P. Wolff. 1994. Measurement of plasma hydroperoxide concentrations by the ferrous-xylenol orange assay in conjunction with triphenylphosphine. *Anal. Biochem.* 220:403–409.

29. Nakamura, T., and H. Maeda. 1991. A simple assay for lipid hydroperoxides based on triphenylphosphine oxidation and high performance liquid chromatography. *Lipids.* 26:765–768.

30. Levi, S., S.J. Yewdall, P.M. Harrison, P. Santambrogio, A. Cozzi, E. Rovida, A. Albertini, and P. Arosio. 1992. Evidence that H- and L-chains have co-operative roles in the iron-uptake mechanism of human ferritin. *Biochem. J.* 288:591–596.

31. Miller, D.M., G.R. Buettner, and S.D. Aust. 1990. Transition metals as catalysts of "autoxidation" reactions. *Free Radic. Biol. & Med.* 8:95–108.

32. Minotti, G., and S.D. Aust. 1992. Redox cycling of iron and lipid peroxidation. *Lipids.* 27:219–226.

33. Minotti, G., and S.D. Aust. 1987. The requirement for Fe(III) in the initiation of lipid peroxidation by Fe(II) and hydrogen peroxide. *J. Biol. Chem.* 262:1098–1104.

34. Crichton, R.R., and R.J. Ward. 1992. Iron metabolism: new perspectives in view. *Biochemistry.* 31:11255–11264.

35. Buege, J.A., and S.D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* 52:302–310.

36. Ryan, T.P., V.M. Samokyszyn, S. Dellis, and S.D. Aust. 1990. Effects of (+)-1,2-bis(3,5-dioxopiperazin-1-yl)propane (ADR-529) on iron-catalyzed lipid peroxidation. *Chem. Res. Toxicol.* 3:384–390.

37. Cecchini, R., O.I. Aruoma, and B. Halliwell. 1990. The action of hydrogen peroxide on the formation of thiobarbituric acid reactive material from microsomes, liposomes or from DNA damaged by bleomycin or phenanthroline. Artefacts in the thiobarbituric acid test. *Free Rad. Res. Commun.* 10:245–258.

38. Boren, J., L. Graham, M. Wettesten, J. Scott, A. White, and S.-O. Olofsson. 1992. The assembly and secretion of apoB 100-containing lipoproteins in Hep G2 cells. *J. Biol. Chem.* 267:9858–9867.

39. Kontron Instruments Analytical Division. 1992. Rapid separation of serum lipoproteins by single-spin vertical discontinuous density gradient ultracentrifugation. Application Note No. 5–92.

40. Frei, B., and J.M. Graziano. 1993. Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibilty of human LDL to metal ion-dependent and -independent oxidation. *J. Lipid Res.* 34: 2135–2145.

41. Minotti, G., A.F. Cavaliere, A. Mordente, M. Rossi, R. Schiavello, R. Zamparelli, and G.F. Possati. 1995. Secondary alcohol metabolites mediate iron delocalization in cytosolic fractions of myocardial biopsies exposed to anticancer anthracyclines. *J. Clin. Invest.* 95:1595–1605.

42. Ulvik, R., and I. Romslo. 1979. Studies on the mobilization of iron from ferritin by isolated rat liver mitochondria. *Biochim. Biophys. Acta.* 588:256–271.

43. Stoscheck, C.M. 1990. Quantitation of Protein. *Methods Enzymol.* 182: 50–68.

44. Luzzago, A., P. Arosio, C. Iacobello, G. Ruggeri, L. Capucci, E. Brocchi, F. De Simone, D. Gamba, E. Gabri, S. Levi, et al. 1986. Immunochemical characterization of human liver and heart ferritins with monoclonal antibodies. *Biochim. Biophys. Acta.* 872:61–71.

45. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

46. Smith, G.N., M. Taj, and J.M. Braganza. 1991. On the identification of a conjugated diene component of duodenal bile as 9Z,11E-octodecadienoic acid. *Free Radical Biol. & Med.* 10:13–21.

47. Corongiu, F.P., G. Poli, M.U. Dianzani, K.H. Cheeseman, and T.F. Slater. 1986. Lipid peroxidation and molecular damage to polyunsaturated fatty acids in rat liver. Recognition of two classes of hydroperoxides formed under conditions in vivo. *Chem. Biol. Interact.* 59:147–155.

48. Reiter, R., and R.F. Burk. 1987. Effect of oxygen tension on the generation of alkanes and malondialdehyde by peroxidizing rat liver microsomes. *Biochem. Pharmacol.* 36:925–929.

49. Slater, T.F. 1984. An overview of methods used for detecting lipid peroxidation. *Methods Enzymol.* 105:283–293.

50. Levi, S., P. Santambrogio, A. Cozzi, E. Rovida, B. Corsi, E. Tamborini, S. Spada, A. Albertini, and P. Arosio. 1994. The role of L-chain in ferritin iron incorporation. Studies of homo and heteropolymers. *J. Mol. Biol.* 238:649–654.

51. Sevanian, A., and E. Kim. 1985. Phospholipase A_2 dependent release of fatty acids from peroxidized membranes. *J. Free Radicals Biol. & Med.* 1:263–271.

52. Morrow, J.D., J.A. Awad, H.J. Boss, I.A. Blair, and L.J. Roberts. 1992. Non-cyclooxygenase-derived prostanoids (F_2 isoprostanes) are formed *in situ* on phospholipids. *Proc. Natl. Acad. Sci. USA.* 89:10721–10725.

53. Girotti, M.J., N. Khan, and B.A. McLellan. 1991. Early measurement of systemic lipid peroxidation products in the plasma of major blunt trauma patients. *J. Trauma.* 31:32–35.

54. Keen, R.R., L. Stella, D.P. Flanigan, and W.E. Lands. 1991. Differential detection of plasma lipid hydroperoxides in sepsis. *Crit. Care Med.* 19:1114–1119.

55. Clot, P., M. Tabone, S. Arico, and E. Albano. 1994. Monitoring oxidative damage in patients with liver cirrhosis and different daily alcohol intake. *Gut.* 35:1637–1643.

56. Carbonneau, M.A., E. Peuchant, D. Sess, P. Canioni, and M. Clerc. 1991. Free and bound malondialdehyde measured as thiobarbituric acid adduct by HPLC in serum and plasma. *Clin. Chem.* 37:1423–1429.

57. Muindi, J.F., H.I. Scher, J.R. Rigas, R.P. Warrell, and C.W. Young. 1994. Elevated plasma lipid peroxide content correlates with rapid plasma clearance of *all-trans* retinoic acid in patients with advanced cancer. *Cancer Res.* 54:2125–2128.

58. Donagh, J., E.T. Fossel, R.L. Kradin, S.M. Dubinett, M. Laposata, and Y.A. Hallaq. 1992. Effects of tumor necrosis factor-α on peroxidation of plasma lipoprotein lipids in experimental animals and patients. *Blood.* 80:3217–3226.

59. Tchekmedyan, N.S., D. Zahyna, C. Halpert, and D. Heber. 1992. Clinical aspects of nutrition in advanced cancer. *Oncology (Basel).* 49(Suppl. 2):3–7.

60. Malvy, D.J., B. Burtschy, J. Arnaud, D. Sommelet, G. Leverger, L. Dostalova, J. Drucker, and O. Amedee-Manesme. 1993. Serum beta-carotene and antioxidant micronutrients in children with cancer. The "Cancer children and antioxidant micronutrients" French Study Group. *Int. J. Epidemiol.* 22: 761–771.

61. Stahelin, B. 1993. Critical re-appraisal of vitamins and trace minerals in nutritional support of cancer patients. *Support. Care Cancer.* 1:295–297.

62. Chautan, M., J. Leonardi, R. Calaf, P. Lechene, R. Grataroli, H. Portugal, A.M. Pauli, H. Lafont, and G. Nalbone. 1992. Heart and liver membrane phospholipid homeostasis during acute administration of various antitumoral drugs to the rat. *Biochem. Pharmacol.* 44:1139–1147.

63. Mustonen, P., and P.K. Kinnunen. 1991. Activation of phospholipase A_2 by adriamycin *in vitro.* Role of drug-lipid interactions. *J. Biol. Chem.* 266:6302–6307.

64. Minotti, G., and S.D. Aust. 1987. An investigation into the mechanism of citrate-Fe(II) dependent lipid peroxidation. *Free Radical Biol. & Med.* 3:379–387.

65. Braughler, J.M., L.A. Duncan, and R.L. Chase. 1986. The involvement of iron in lipid peroxidation: importance of ferric to ferrous ratios in initiation. *J. Biol. Chem.* 261:10282–10289.

66. Goddard, J.G., and G.D. Sweeney. 1987. Delayed ferrous iron-dependent peroxidation of rat liver microsomes. *Arch. Biochem. Biophys.* 259:372–381.

67. Ursini, F., M. Maiorino, P. Hochstein, and L. Ernster. 1989. Microsomal lipid peroxidation: mechanisms of initiation. *Free Radical Biol. & Med.* 6:31–36.

68. Ko, K.M., and D.V. Godin. 1990. Ferric iron dependent lipid peroxidation in erythrocyte membranes: effects of phytic acid and butylated hydroxytoluene. *Mol. Cell. Biochem.* 95:125–131.

69. Kukielka, E., and A.I. Cederbaum. 1990. NADPH- and NADH- dependent oxygen radical generation by rat liver nuclei in the presence of redox cycling agents and iron. *Arch. Biochem. Biophys.* 283:326–333.

70. Rohn, T.T., T.R. Hinds, and F.F. Vincenzi. 1993. Ion transport ATPases as targets for free radical damage. Protection by an aminosteroid of the Ca^{2+} pump ATPase of human red blood cell membrane. *Biochem. Pharmacol.* 46: 525–534.

71. Minotti, G. 1990. NADPH- and adriamycin-dependent microsomal release of iron and lipid peroxidation. *Arch. Biochem. Biophys.* 277:268–276.

72. Wissel, P.S., R.A. Galbraith, S. Sassa, and A. Kappas. 1988. Tin-protoporphyrin inhibits heme oxygenase and prevents the decline in hepatic heme and cytochrome P-450 contents produced in nude mice by tumor transplantation. *Biochem. Biophys. Res. Commun.* 150:822–827.

73. Seiser, C., S. Teixeira, and L.C. Kuhn. 1993. Interleukin-2–dependent transcriptional and post-transcriptional regulation of transferrin receptor mRNA. *J. Biol. Chem.* 268:13074–13080.

74. Minotti, G., and M. Ikeda-Saito. 1992. Fe(II) oxidation and Fe(III) incorporation by the M_r 66,000 Microsomal Iron Protein that stimulates NADPH oxidation. *J. Biol. Chem.* 267:7611–7614.

75. Rothman, R.A., J.A. Serroni, and J.L. Farber. 1992. Cellular pool of transient ferric iron, chelatable by desferrioxamine and distinct from ferritin, that is involved in oxidative injury. *Mol. Pharmacol.* 42:703–710.

76. deSilva, D., and S.D. Aust. 1992. Stoichiometry of Fe(II) oxidation during ceruloplasmin-catalyzed loading of ferritin. *Arch. Biochem. Biophys.* 298: 259–264.

77. Minotti, G. 1989. Adriamycin-dependent release of iron from microsomal membranes. *Arch. Biochem. Biophys.* 268:398–403.

78. Weitzman, S.A., B. Lorell, R.W. Carey, S. Kaufman, and T.P. Stossel. 1980. Prospective studies of tocopherol prophylaxis for anthracycline cardiac toxicity. *Am. J. Cardiol.* 56:157–161.

79. Myers, C., R. Bonow, S. Palmieri, J. Jenkins, B. Corden, G. Locker, J. Doroshow, and S.A. Epstein. 1983. A randomized controlled trial assessing the prevention of doxorubicin cardiomyopathy by *N*-acetylcysteine. *Sem. Oncol.* 10(Suppl.):53–55.

80. Muggia, F.M. 1994. Cytoprotection: concepts and challenges. *Support. Care Cancer.* 2:377–379.

81. Malisza, K.L., and B.B. Hasinoff. 1995. Doxorubicin reduces the iron (III) complexes of the hydrolysis products of the antioxidant cardioprotective agent dexrazoxane (ICRF-187) and produces hydroxyl radicals. *Arch. Biochem. Biophys.* 316:680–688.