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Oxidative Degradation of Cardiotoxic Anticancer Anthracyclines to Phthalic Acids

NOVEL FUNCTION FOR FERRYLMYOGLOBIN*

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We show that the pseudoperoxidase activity of ferrylmyoglobin (Mb^{IV}) promotes oxidative degradation of doxorubicin (DOX), an anticancer anthracycline known to induce severe cardiotoxicity. Mb^{IV}, formed *in vitro* by reacting horse heart Mb^{III} with H_2O_2, caused disappearance of the spectrum of DOX at 477 nm and appearance of UV-absorbing chromophores that indicated opening and degradation of its tetracyclic ring. Electron spray ionization mass spectrometry analyses of DOX/Mb^{IV} ultrafiltrates showed that DOX degradation resulted in formation of 3-methoxyphthalic acid, the product of oxidative modifications of its methoxy-substituted ring D. Other methoxy-substituted anthracyclines similarly released 3-methoxyphthalic acid after oxidation by Mb^{IV}, whereas demethoxy analogs released simple phthalic acid. Kinetic and stoichiometric analyses of reactions between DOX and Mb^{III}/H_2O_2 or hemin/H_2O_2 showed that the porphyrin radical of Mb^{IV}-compound I and the iron-oxo moiety of Mb^{IV}-compound II were sequentially involved in oxidizing DOX; however, oxidation by compound I formed more 3-methoxyphthalic acid than oxidation by compound II. Sizeable amounts of 3-methoxyphthalic acid were formed in the heart of mice treated with DOX, in human myocardial biopsies exposed to DOX *in vitro*, and in human cardiac cytosol that oxidized DOX after activation of its endogenous myoglobin by H_2O_2. Importantly, H9c2 cardiomyocytes were damaged by low concentrations of DOX but could tolerate concentrations of 3-methoxyphthalic acid higher than those measured in murine or human myocardium. These results unravel a novel function for Mb^{IV} in the oxidative degradation of anthracyclines to phthalic acids and suggest that this may serve a salvage pathway against cardiotoxicity.

Myoglobin (Mb)[1] has been implicated as a potential catalyst of cardiac damage induced by increased formation of hydrogen peroxide (H_2O_2). Under normal conditions the majority of Mb is found in its oxygenated form (MbO_2), which interacts slowly with H_2O_2 (k = 20.8 s^{-1} M^{-1}); however, both deoxy-Mb^{II} and metmyoglobin (Mb^{III}) react rapidly with H_2O_2 ($k = 3.6 \times 10^3$ and 3.4×10^4 s^{-1} M^{-1}, respectively) (1, 2). An ideal setting for reactions between Mb and H_2O_2 has therefore been identified in cardiac ischemia-reperfusion, a condition characterized by conversion of $Mb^{II}O_2$ to Mb^{III}/deoxy-Mb^{II} during ischemia and by formation of H_2O_2 during blood reflow (3, 4). Hydrogen peroxide causes two-equivalent oxidation of Mb^{III} to ferrylmyoglobin (Mb^{IV}), a hypervalent species that oxidizes polyunsaturated fatty acids and several other biomolecules in a fashion similar to that described for the compound I or II of peroxidases. The reaction sequence through which Mb^{IV} is generated from Mb^{III} has been considered as shown in Reactions 1 and 2,

$$\text{Por-Mb}^{III}\text{-globin} + H_2O_2 \rightarrow \text{Por}^{\cdot}\text{-Mb}^{IV}\text{-globin} + H_2O$$

REACTION 1

$$\text{Por}^{\cdot}\text{-Mb}^{IV}\text{-globin} \rightarrow \text{Por-Mb}^{IV}\text{-globin}^{\cdot}$$

REACTION 2

In Reaction 1, H_2O_2 converts Mb^{III} to a compound I-like species in which both oxidizing equivalents are retained in the heme pocket, one in the form of a long lived iron-oxo moiety (Fe^{IV}=O) and the other in the form of a transient porphyrin π-cation radical ($Por^{\cdot}$) (5). In Reaction 2, the porphyrin radical dissipates in the globin, causing formation of amino acid radicals while leaving the heme moiety in a Fe^{IV}=O form similar to compound II (5–8).

We developed an interest in possible reactions between Mb and doxorubicin (DOX), an anticancer anthracycline which exhibits activity against several tumors but also causes severe cardiotoxicity. The rationale for investigating DOX-Mb interactions was offered by several considerations. On the one hand, cyclic reduction-oxidation of a quinone moiety in the tetracyclic ring of DOX (Fig. 1) generates H_2O_2 in excess of the detoxifying capacity of cardiomyocytes (9–11). On the other hand, DOX causes a 4-fold stimulation of the autoxidation of $Mb^{II}O_2$ to Mb^{III} (12) and inhibits Mb^{III} reductases that would regenerate

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[1] The abbreviations used are: Mb, myoglobin; DOX, doxorubicin; Mb^{III}, metmyoglobin; Mb^{IV}, ferrylmyoglobin; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; LPO, lactoperoxidase; DNR, daunorubicin; IDA, idarubicin; ABTS, 2,2′-diazinobis(3-ethylbenzothiazoline-6-sulfonic acid); ESI-MS, electron spray ionization-mass spectrometry; TIC, total ion count; C. V., cone voltage; M, molecular ion; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; HPLC, high pressure liquid chromatography.

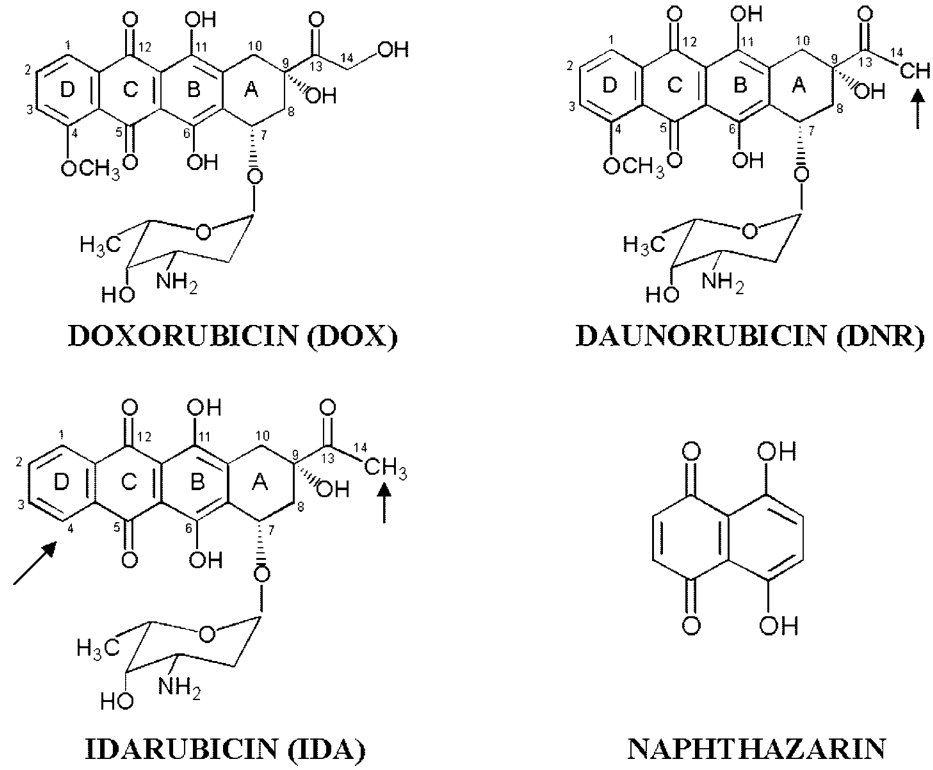

FIG. 1. **Structures of DOX and analogs used in this study.** The *arrows* indicate structural differences between DNR or IDA and DOX (DNR, presence of a side chain methyl terminus in place of a primary alcohol; IDA, same as DNR and lack of a methoxy residue in ring D).

$Mb^{II}O_2$ (13). Thus, several factors seem to enable DOX to promote reactions between Mb^{III} and H_2O_2, possibly exposing cardiomyocytes to lipid peroxidation or other forms of oxidative injury induced by Mb^{IV}. In contrast to these premises, however, we found that DOX inhibited lipid peroxidation induced by Mb^{IV} in reconstituted chemical models (14). We demonstrated that the "antioxidant" effect of DOX was due to its ability to reduce Mb^{IV} back to Mb^{III}, a process mediated by a hydroquinone in juxtaposition to the quinone (*cf.* Fig. 1) (14). We also noticed that electron transfer from DOX to Mb^{IV} was accompanied by a loss of the optical and fluorescent properties of the anthracycline, as if DOX underwent opening and degradation of its tetracyclic ring (14). These results provided unexpected evidence that DOX could both favor Mb^{IV} formation and prevent oxidative damage by serving itself as a suicide substrate for Mb^{IV}.

Attempts to identify the product(s) of Mb^{IV}-dependent DOX degradation were unsuccessful (14), and similar problems were encountered by other investigators when DOX was oxidized with horseradish peroxidase, microperoxidase 11 (product of the proteolytic digestion of cytochrome *c*), or $^{\bullet}NO_2$ radicals generated through lactoperoxidase/NO_2^- or myeloperoxidase/NO_2^- (15–18). Lack of information about the nature of degradation products precludes an appraisal of the mechanisms and consequences of Mb^{IV}-anthracycline interactions. Therefore, we designed experiments to elucidate how Mb^{IV} oxidizes DOX and to identify the product(s) of DOX degradation. Moreover, we characterized the possible role of degradation product(s) in anthracycline-induced cardiotoxicity.

EXPERIMENTAL PROCEDURES

Chemicals—Doxorubicin, daunorubicin (DNR), and 4-demethoxydaunorubicin (idarubicin, IDA) were obtained through the courtesy of Pharmacia-Upjohn, Milan, Italy. Thymol-free bovine liver catalase, horse heart Mb^{III}, hemin, type VI-A horseradish peroxidase (HRP), bovine milk lactoperoxidase (LPO), 2,2′-diazinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), naphthazarin (5,8-dihydroxy-1,4-naphthoquinone), and all other chemicals were from Sigma. 3-Methoxyphthalic acid was synthesized by oxidation of 2,3-dimethylanisole with a large excess of potassium permanganate (19) and purified by crystallization from water.

Mb^{IV} Formation—Mb^{IV} was formed by reacting Mb^{III} with a 2-fold excess of H_2O_2. As shown previously (14), this H_2O_2:Mb^{III} ratio gives complete oxidation of Mb^{III} to Mb^{IV} while also avoiding confounding factors due to the possible release of iron from the heme pocket. Mb^{III} was quantitated by assuming $\epsilon_{630\ nm} = 3.5\ mM^{-1}\ cm^{-1}$; Mb^{IV} was quantitated according to the formula: Mb^{IV} (mM) = $((249 \times A_{550\ nm}) - (367 \times A_{630\ nm}))$ (20). Unless otherwise indicated, the experiments were carried out at 37 °C in 0.3 M NaCl, carefully adjusted to pH 7.0. This was done because we noticed that common buffers like phosphate or Tris or Hepes accelerated a spontaneous decay of Mb^{IV} to Mb^{III}; buffers are also known to participate in anthracycline redox reactions (21). Although unbuffered, the pH of incubations did not vary throughout the experiment.

Spectrophotometric Assays for Mb^{IV}-dependent DOX Degradation—After correction of Mb^{III} absorbance, known amounts of DOX were added, and the spectrum of the anthracycline at 477 nm was recorded immediately. Next, H_2O_2 was added, and the disappearance of the spectrum of DOX was monitored at regular times. Similar settings were adopted when hemin was used in place of Mb^{III}; in the latter case 10 mM stock solutions of hemin were prepared in 100 mM NaOH and diluted appropriately with 50 mM phosphate buffer to obtain 1 mM working solutions.

Spectrophotometric Assays for DOX-dependent Mb^{IV} Reduction—In the experiments described in the preceding section, involving sequential additions of Mb^{III} and DOX and H_2O_2, stepwise H_2O_2-dependent oxidation of Mb^{III} to Mb^{IV}/Fe^{IV}=O and DOX-dependent reduction of Mb^{IV}/Fe^{IV}=O to Mb^{III} were monitored at 426 or 410 nm, respectively (6). In other experiments, Mb^{III} was added, and its spectrum (peaks at 502 and 630 nm) was recorded. Next, H_2O_2 was added, and the spectrum of Mb^{IV}/Fe^{IV}=O (peaks at 546 and 586 nm) was taken at regular times until DOX was included. Doxorubicin-dependent Mb^{IV}/Fe^{IV}=O reduction and Mb^{III} regeneration were eventually monitored as disappearance of peaks at 546 and 586 nm or reappearance of peaks at 502 and 630 nm, respectively.

HPLC-UV-ESI(+) MS Analysis of DOX Degradation—Varying amounts of DOX were incubated with equimolar Mb^{III} and a 2-fold excess of H_2O_2. After 10 min the reaction mixtures were added with catalase (2600 units) to decompose any residual H_2O_2 and ultrafiltered through YM 10 Centricon® (Millipore Corp., Bedford, MA). Myoglobin-free ultrafiltrates were injected without further manipulations into a BioSys 510 Liquid Chromatography System (Beckman Instruments, Fullerton, CA) equipped with a (1 × 20 cm) Ultrogel Ac34 column

(BioSepra SA, Villeneuve la Garenne Cedex, France) equilibrated with 2.5 mM NaCl, 2.5 mM CH_3COONH_4. Samples were eluted with the same medium at the flow rate of 0.5 ml/min. UV-absorbing fractions were pooled, passed through Anotop 25® filters (Merck), and concentrated by evaporation. After suspension in a minimum volume of CH_3OH, the sample was laid into a Waters 2790 separation module (alliance HT) equipped with a Waters 996 photodiode array and a ZMD single quadrupole mass spectrometer (Micromass, UK) as detectors in series. Reversed phase HPLC was performed using a LiChrospher® (Merck) analytical column (100 RP-18 (5 μm), 150 × 4 mm) and a mobile phase composed of (H_2O + 0.1% trifluoroacetic acid):(CH_3CN + 0.1% trifluoroacetic acid) at an initial ratio of 80:20, which was decreased to 30:70 in 15 min and returned to initial conditions in 20 min, at the flow rate of 0.5 ml/min. After chromatography and photodiode array detection (210–600 nm), mass spectra were acquired at low (unity) resolution in series at a rate of 300 atomic mass unit/s in compressed centroid mode. Standard ESI ion source, operated in positive ion mode, was used with capillary voltage of 3.25 kV and cone voltage (C.V.) of 20 or 50 V to obtain mass spectra under low or high energy conditions. The source and desolvation temperatures were 100 and 200 °C, respectively. Nitrogen was used as the nebulizer gas at a flow rate of 59 liters/h and as the desolvation gas at a flow rate of 430 liters/h.

Liquid Chromatography-Tandem Mass Spectrometry—Because HPLC-UV-ESI(+) MS identified 3-methoxyphthalic acid (molecular mass = 196 daltons) as a possible product of Mb^{IV}-dependent DOX degradation, we used liquid chromatography-tandem mass spectrometry to further characterize DOX/Mb^{IV} ultrafiltrates in comparison with authentic 3-methoxyphthalic acid. Reversed phase HPLC was carried out using a Waters Alliance 2795 separation module and an Agilent Hypersil ODS® column (2.1 × 250 mm; C_{18} 5 μm, 120 Å). Ten microliter samples were eluted at a flow rate of 200 μl/min with (H_2O + 0.2% HCOOH) − (10% CH_3CN + 0.2% HCOOH). After 2 min CH_3CN was increased linearly to reach 70% in 6 min and was then maintained isocratically for 4 more min. The HPLC system was directly coupled with a Micromass Quattro Ultima™ triple quadrupole mass spectrometer equipped with a Z-Spray ESI source, operated in negative ion mode to improve sensitivity. Analytical conditions were optimized with direct infusion of standard solutions of 4.5 μM 3-methoxyphthalic acid, dissolved in $H_2O{\cdot}CH_3CN$ (50:50, v/v), 0.2% HCOOH, and eluted at a flow rate of 5 μl/min. The product ion scan mode for tandem MS was used. The mass spectrometer monitored the deprotonated molecule $[M - H]^-$ of 3-methoxyphthalic acid at m/z 195 via the first quadrupole filter, and collision-induced dissociation was performed at the second quadrupole (collision gas, argon, at 2.4e-3 mbar, and collision energy at 8 eV). The daughters of m/z 195 were monitored via the third quadrupole in the mass range of 50–300 atomic mass units. Spectra were acquired in the continuous mode. The capillary and cone voltages were set at −3 kV and −40 V, respectively. The source and desolvation temperatures were set at 110 and 400 °C, respectively, and the desolvation gas (N_2) flow was set at 600 liters/h. Cone gas (N_2) was not used, whereas the nebulizer gas (N_2) was left at its maximum flow.

Other HPLC Assays for Anthracyclines and Phthalates—Anthracycline/Mb^{III}/H_2O_2 incubations were extracted with a 4-fold excess of $CHCl_3$/CH_3OH (1:1) and subjected to 10 min of low speed centrifugation. Next, 20 μl of the methanolic phase were analyzed for 3-methoxyphthalic acid or simple phthalic acid by reversed phase HPLC using an HP 1100 system (Hewlett-Packard Co., Palo Alto, CA). Chromatography was performed using a Hewlett-Packard Zorbax CN column (250 × 4.6 mm, 5 μm) operated at 25 °C. Samples were eluted at the flow rate of 1.5 ml/min by using a 15-min linear gradient from 50 mM NaH_2PO_4 to CH_3CN, 25 mM NaH_2PO_4, all adjusted to pH 4.0 with orthophosphoric acid and filtered through a 0.22-μm membrane (Millipore Corp.). Phthalates were detected at 300 nm by on-line diode array spectrometer. Identification was obtained by co-elution with authentic standards and ESI(+)-MS analysis of peak fractions; quantification was obtained against standard curves prepared with known amounts of phthalates subjected to the same extraction and chromatographic procedures. Retention times of 3-methoxyphthalic acid or simple phthalic acid were 4.1 and 4.7 min, respectively. Anthracyclines were detected by diode array and fluorescence spectroscopy (excitation at 470 nm, emission at 550 nm); retention times of DOX, DNR, IDA, and naphthazarin were 12.1, 13.8, 14.3, or 14.5 min, respectively. Quantification was obtained against appropriate standard curves of each anthracycline.

3-Methoxyphthalic Acid Formation in DOX-treated Mice—Two- to three-month-old male Balb/c mice, weighing 20–30 g, were treated with DOX (10 mg/kg, intravenously) and sacrificed after 4 or 24 h. Heart, liver, and kidneys were removed, and 0.2-g aliquots were homogenized in ice-cold 0.3 M NaCl. Next, the homogenates were added with 5 ml of ice-cold acetone, incubated at −20 °C for 30 min, and centrifuged to precipitate proteins. The supernatants were vacuum-dried, cleared of gross particulates by low speed centrifugation in CH_3OH, and assayed for DOX and 3-methoxyphthalic acid by HPLC. Fluorescent anthracycline metabolites, like the C-13 secondary alcohol metabolite and hydroxy- or deoxyaglycones, were also measured using appropriate standards (retention times: 10.9, 12.5, and 13.8 min, respectively).

3-Methoxyphthalic Acid Formation in Human Myocardium—Small myocardial samples were taken from the lateral aspect of excluded right atrium of patients undergoing aorto-coronary bypass grafting. All specimens were routinely disposed of by the surgeons during cannulation procedures; therefore, patients were not subjected to any unjustified or ethically unacceptable loss of tissue (22). Thin myocardial strips (~0.1 g) were dissected and placed in 5 ml of Krebs bicarbonate buffer in the presence of 1–10 μM DOX. After 4 h at 37 °C the strips were removed, washed extensively in ice-cold 0.3 M NaCl, and extracted for HPLC analysis of DOX and fluorescent metabolites or 3-methoxyphthalic acid as described in the preceding section. In other experiments, pools of 10–15 anonymous biopsies were processed by sequential homogenization, 20-min centrifugations at 16,000 and 25,000 × g, and 90-min ultracentrifugation at 105,000 × g. The supernatant (cytosol) was used immediately or after precipitation with 65% ammonium sulfate, a procedure known to remove Mb by "salting out" (4, 14). Mb-containing or Mb-depleted cytosol (referred to as Mb^+ or Mb^- cytosol, respectively) was eventually dialyzed against two 1-liter changes of 0.3 M NaCl, 1 mM EDTA to remove adventitious iron and other low molecular weight contaminants, and then against two 1-liter changes of 0.3 M NaCl to remove EDTA-iron complexes (23). Based on nephelometric and spectral assays by us and others (24), the myoglobin content of right atrium homogenates and Mb^+ or Mb^- cytosol was determined to be 1.3 and 2.9 or 0.6 nmol/mg of protein, respectively. Whole homogenates and Mb^+ or Mb^- cytosol were eventually reconstituted with known amounts of DOX and H_2O_2 and assayed for anthracycline degradation and 3-methoxyphthalic acid formation by HPLC.

Experiments with Isolated Cardiomyocytes—The embryonic rat heart-derived cell line H9c2 (American Type Culture Collection (CRL 1446)) was grown in Dulbecco's modified Eagle's medium as described previously (25). Subconfluent cells were seeded in quadruplicate in 24-well plates (~10^5 cells/well) and incubated overnight in the absence or presence of increasing amounts of DOX or 3-methoxyphthalic acid. At the end of treatment, cell viability was measured by 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) conversion assay as an indicator of mitochondrial function (Sigma kit) (26)

Other Conditions and Assays—Proteins were assayed by the bicinchoninic acid method (27). The peroxidative activity of HRP, LPO, Mb^{III} and hemin was determined by monitoring oxidation of ABTS to its cation radical $ABTS^{\cdot +}$ ($\epsilon_{660\ nm} = 12 \times 10^3\ M^{-1}\ cm^{-1}$) (16). All values are given as means ± S.E.; where indicated, data were analyzed by unpaired Student's t test, and differences were considered significant when p was < 0.05. Other conditions are indicated in the legends to figures and tables.

RESULTS

Mb^{IV}-dependent DOX Degradation, Role of Compound I and II—Incubation of DOX with H_2O_2 and Mb^{III} resulted in a time-dependent decay of the spectrum of the anthracycline and concomitant development of absorbance at 350 nm (Fig. 2*A*). These spectral changes were similar to those observed by others when monitoring reactions of DOX with H_2O_2 and HRP or lactoperoxidase (16); under those conditions the loss of absorbance at 477 nm was attributed to degradation of the anthraquinone chromophore, whereas the increase of absorbance at 350 nm was attributed to accumulation of degradation product(s). In our study putative product(s) of anthracycline degradation were found to exhibit more complex characteristics. In fact, difference spectra obtained by subtracting the initial scan of DOX/Mb^{III} mixtures to the scans obtained at regular times after H_2O_2 addition revealed that the increase in absorbance at 350 nm was accompanied by additional peaks at ~210, 242, and 280 nm, possibly reflecting the formation and spectral overlapping of a complex mixture of degradation products (Fig. 2*B*). Doxorubicin degradation increased with the ratio of Mb^{IV} to DOX, near-to-complete degradation occurring at Mb^{IV}:DOX ratios around unity (Fig. 2*C*). Mb^{IV} was less effective than HRP

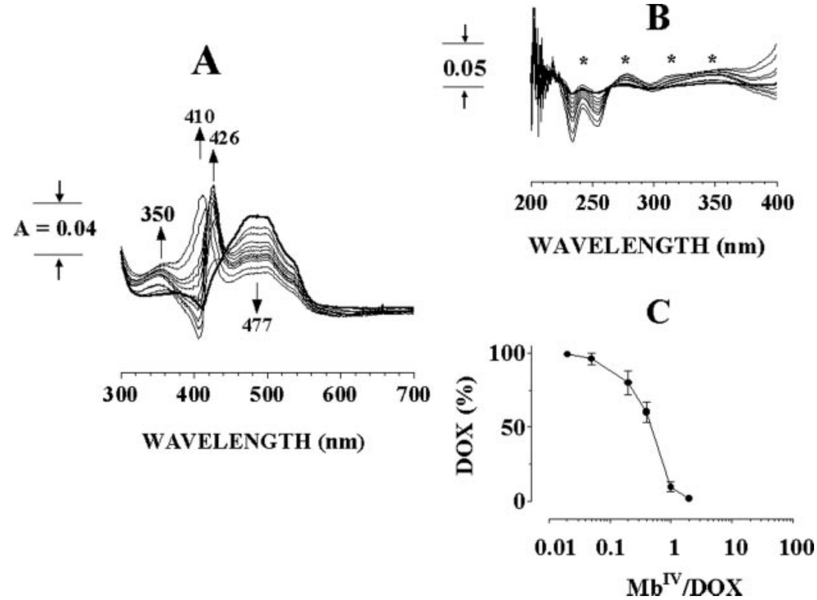

FIG. 2. **Mb^{IV}-dependent DOX degradation.** *A*, incubations (1 ml final volume) contained 3 μM Mb^{III} in 0.3 M NaCl, pH 7.0, 37 °C. After correction for Mb^{III} absorbance, 5 μM DOX was added, and its spectrum was recorded immediately. Next, 6 μM H_2O_2 was added, and spectra were recorded every minute. All samples were corrected for background absorbance of reference cuvettes lacking DOX. *B*, difference spectra obtained by subtracting the spectrum of unchanged DOX to the scans recorded at different times after H_2O_2 addition; the *boldface trace* is the difference spectrum recorded 1 min after H_2O_2 addition. *C*, increasing Mb^{IV}:DOX ratios were obtained by incubating 5 μM DOX with 0.1–10 μM Mb^{III}, always reacted with a 2-fold excess of H_2O_2. Values are means ± S.E. of three determinations; values without vertical bars have S.E. within the symbols.

or LPO at oxidizing a typical peroxidatic substrate like ABTS, its k_{cat}/k_m for formation of $ABTS^{\cdot+}$ being ~2 orders of magnitude lower than that determined for either peroxidase (1.8 ± 0.2 × 10^3 for Mb^{IV} *versus* 7 ± 0.4 × 10^5 or 1.1 ± 0.3 × 10^5 M^{-1} s^{-1}, respectively; n = 3). However, Mb^{IV} was more effective than HRP or LPO at degrading DOX, its k_{cat}/k_m for DOX disappearance being ~1 order of magnitude higher than that determined for either peroxidase (1.1 ± 0.2 × 10^3 *versus* 0.9 ± 0.1 × 10^2 or 3 ± 0.2 × 10^2 M^{-1} s^{-1}, respectively; n = 3). Thus, a pseudoperoxidase like Mb^{IV} was more effective than authentic peroxidases at inducing oxidative degradation of DOX.

Incubation of DOX with H_2O_2 and Mb^{III} was also accompanied by appearance of peaks at 426 and 410 nm. The peak at 426 nm developed immediately after the addition of H_2O_2; it reflected oxidation of Mb^{III} with H_2O_2 and consequent formation of Fe^{IV}=O (6). The peak at 410 nm developed later during the course of the reaction and eventually replaced the peak at 426 nm; it reflected reduction of Fe^{IV}=O back to Mb^{III} (6). These results suggested that DOX degraded by oxidizing with Fe^{IV}=O/compound II, but the kinetics of DOX degradation did not match the kinetics of Fe^{IV}=O reduction/Mb^{III} regeneration. In fact, the absorbance of DOX at 477 nm decreased immediately after mixing Mb^{III} with H_2O_2 and dropped to ~60% its initial level in ~4 min, *i.e.* the time when Fe^{IV}=O was at its maximum detectable level (Fig. 3, *A* and *B*). Based upon these findings, we characterized whether DOX degradation was initiated by a compound I involving Fe^{IV}=O and a porphyrin radical. To obtain this information, we incubated DOX with H_2O_2/hemin, an established source of Fe^{IV}=O/porphyrin radical (28). Preliminary experiments showed that hemin was less effective than equimolar Mb^{III} at catalyzing ABTS oxidation in the presence of H_2O_2, even when H_2O_2:hemin ratios were much higher than H_2O_2:Mb^{III} ratios (units of peroxidative activities (nmol $ABTS^{\cdot+}$/nmol heme/min): 0.02 for 20:1 H_2O_2-hemin *versus* 0.4 for 2:1 H_2O_2-Mb^{III}). Despite its reduced peroxidative activity, hemin/H_2O_2 was quite effective at degrading DOX, giving the same spectral changes obtained with Mb^{III}/H_2O_2 (loss of absorbance at 477 and formation of multiple peaks at ~210, 242, 280, and 350 nm; Fig. 4*A* and *inset*). Doxorubicin degradation actually occurred much faster with hemin-H_2O_2 than with Mb^{III}-H_2O_2. This was observed when DOX was incubated with equimolar Mb^{III} or hemin, and anthracycline degradation was normalized to peroxidative activities (Fig. 4*B*). It was also observed when the concentrations of H_2O_2 and Mb^{III} or hemin were adjusted to achieve similar peroxidative activities in the incubations (Fig. 4*C*). The anthracycline-degrading activity of hemin/H_2O_2 could not be attributed to a denaturation of the heme pocket and consequent release of redox-active iron due to the high H_2O_2:hemin ratios used in this study; in fact, DOX underwent degradation also in the presence of EDTA (*cf.* Fig. 4, *B* and *C*). Thus, DOX oxidative degradation was initiated by the porphyrin radical of compound I, followed by the action of Fe^{IV}=O/compound II.

HPLC-UV-ESI(+)-MS Characterizations of DOX Degradation Products—Previous ESI and MALDI mass spectral analyses of organic extracts derived from peroxidase-anthracycline incubations did not reveal anything but reduced intensity or disappearance of molecular ions attributable to intact anthracyclines or fragments generated under high energy conditions; unambiguous evidence for newly formed degradation products was not obtained (16). In attempting characterization of products of Mb^{IV}-dependent DOX degradation, we therefore considered that it was necessary to avoid organic extractions or other extensive manipulations that might have caused a loss of products with unknown polarity and partitioning in laboratory solvents. We developed a flow chart in which Mb^{IV}/DOX incubations were subjected to ultrafiltration in place of organic extractions. Next, the ultrafiltrates were subjected to gel permeation under low ionic strength conditions (2.5 mM NaCl, 2.5 mM CH_3COONH_4), a procedure needed to reduce the concentration of NaCl in the samples and to avoid interferences of Na^+ clusters with ESI-MS analyses (*cf.* "Experimental Procedures").[2] This procedure enabled us to isolate a gross fraction that eluted before DOX, possibly due to micelle formation and/or gel electrostatic repulsions, and exhibited the same retention time when the eluant was monitored at 210, 242, 280, or 350 nm (*i.e.* the UV peaks of Mb^{IV}- or H_2O_2/hemin-degraded

[2] In preparing for analyses of DOX degradation product(s), we acquired ESI(+)-MS spectra of DOX standards subjected to the same ultrafiltration/gel permeation procedure and dissolved in a (1:1) H_2O:CH_3CN mixture containing 2 mM CH_3COONH_4. Low energy mass spectrum showed a quasi-molecular ion at m/z 544 $[M + H]^+$, whereas in the high energy mass spectrum several fragments were observed: m/z 415 (aglycone), 397 (aglycone without one water molecule), 379 (aglycone with aromatized ring A), 361 (probably aromatized aglycone without −OH in the side chain), 321 (aromatized aglycone without side chain), 148 (protonated sugar), and 130 (sugar without −OH in anomeric position) (see also Refs. 16, 29, and 30).

DOX). The amplitude of this peak increased with the amount of DOX that had been reacted with Mb^{III}/H_2O_2, further indicating that it derived from Mb^{IV}-degraded DOX (Fig. 5*A*). Reversed phase HPLC resolved this material into several peaks, as shown in the chromatogram obtained at $\lambda_{210-600\ nm}$ (Fig. 5*B*). Among these peaks only that corresponding to a retention time of 5.02 min gave also a significant total ion count (TIC), which was detected at a retention time of 5.21 min in the ESI-positive mass spectra acquisition (Fig. 5*C*). The slight peak-to-peak delay (from 5.02 to 5.21 min) was due to the dead volume between photodiode array and mass spectrometer.

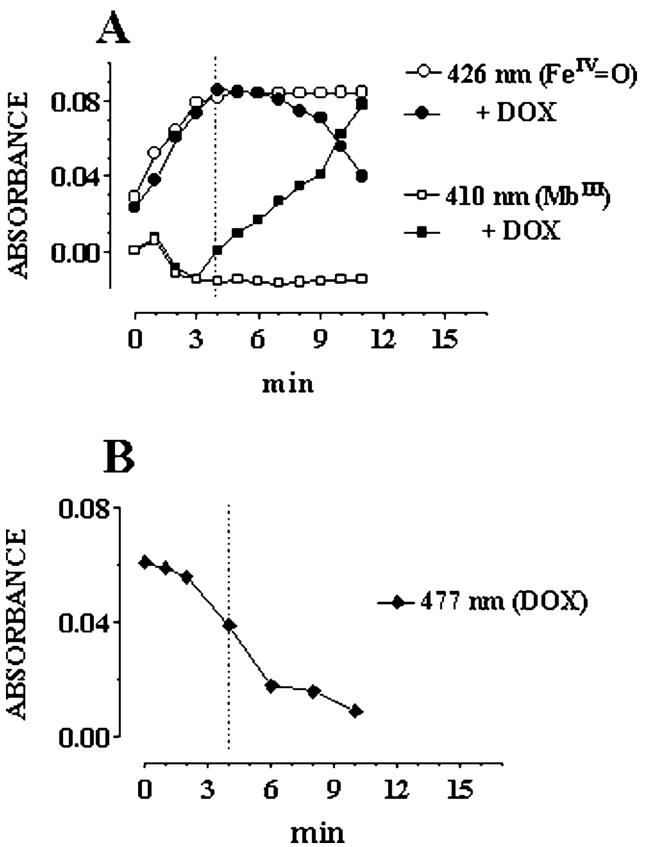

FIG. 3. **Time courses of Fe^{IV}=O formation, Mb^{III} regeneration, and DOX degradation.** Incubations and spectral analyses were as described in Fig. 2*A*, and absorbances at 410, 426 (*A*), and 477 nm (*B*) were monitored simultaneously.

Because mass spectra acquisition is fundamental for characterizing molecules, these conditions gave us an opportunity to identify only one component of the complex material analyzed. As also shown in Fig. 5, the UV spectrum of the unknown compound was characterized by a single maximum at 299 nm and no absorbance over 350 nm. Under low energy conditions (C.V. = 20 V) the mass spectrum displayed a quasi-molecular ion $[M + H]^+$ at *m/z* 197, indicating a possible molecular mass of 196 daltons for the molecule; under high energy conditions (C.V. = 50 V) a main fragment at *m/z* 179 was recorded, possibly indicating the loss of water $[M + H - 18]^+$ from the quasi-molecular ion. Based upon these mass spectra, and taking the structure of DOX into account, we tentatively assigned the unknown molecule to 3-methoxyphthalic acid, product of oxidative modifications of ring D of DOX. Indirect support to such conclusion was also offered by the striking similarity between the UV spectrum of the unknown molecule and that reported for 3-methoxyphthalic acid in ethanol (λ_{max} = 298 nm) (31). Moreover, (i) authentic 3-methoxyphthalic acid co-chromatographed with the ultrafiltrates of Mb^{IV}/DOX in gel permeation while also giving the same retention time in HPLC/ESI(+)-MS; (ii) the UV spectrum of HPLC-eluted 3-methoxyphthalic acid exhibited the same λ_{max} = 299 nm of the spectrum of the unknown compound; (iii) the low energy mass spectrum of 3-methoxyphthalic acid gave the expected quasi-molecular ion $[M + H]^+$ at *m/z* 197, and the high energy mass spectrum gave a fragment at *m/z* 179 (Fig. 6). This fragment ion, similar to that observed when recording a mass spectrum of the unknown compound under high energy, was therefore consistent with H_2O elimination from protonated 3-methoxyphthalic acid and with the possible formation of its protonated anhydride (see also Fig. 6).

LC-ESI(−) MS/MS of 3-Methoxyphthalic Acid and Mb^{IV}-degraded DOX—To strengthen identification of 3-methoxyphthalic acid as a product of DOX degradation, we characterized a more detailed fragmentation pattern of DOX/Mb^{IV} ultrafiltrates *vis à vis* authentic 3-methoxyphthalic acid. For this purpose we used LC-ESI MS/MS in product ion scan mode. Under the analytical conditions adopted in our study (*cf.* "Experimental Procedures"), the negative ion mode proved to be

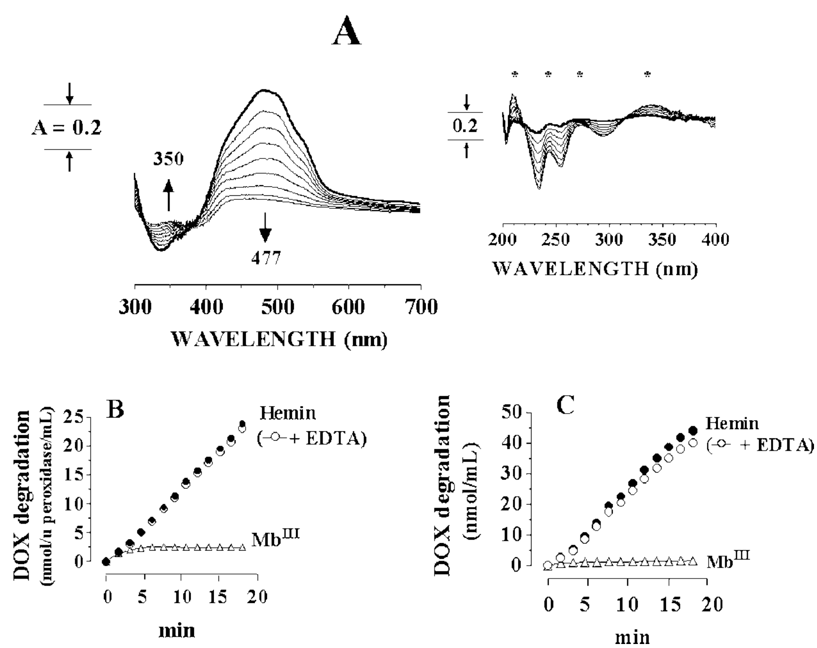

FIG. 4. **Hemin-dependent DOX degradation.** *A*, incubations (1 ml final volume) contained DOX and hemin (both 50 μM); reactions were started by adding H_2O_2 (1 mM), and spectra were taken every 90 s. The *inset* shows spectra obtained by subtracting the spectrum of unchanged DOX to the scans recorded at different times after H_2O_2 addition; the *boldface trace* is the difference spectrum recorded 90 s after H_2O_2 addition. *B*, incubations contained 25 μM DOX and equimolar Mb^{III} or hemin; reactions were started by adding H_2O_2 at 2 or 20:1 ratios to Mb^{III} or hemin, respectively, and rates of DOX degradation were normalized to peroxidative activities. *C*, incubations contained 50 μM DOX and either 2.5 μM Mb^{III} or 50 μM hemin, corresponding to 1 unit of peroxidative activity/ml; reactions were started by adding H_2O_2 at 2 or 20:1 ratios to Mb^{III} or hemin, respectively. Where indicated incubations contained 0.1 mM EDTA.

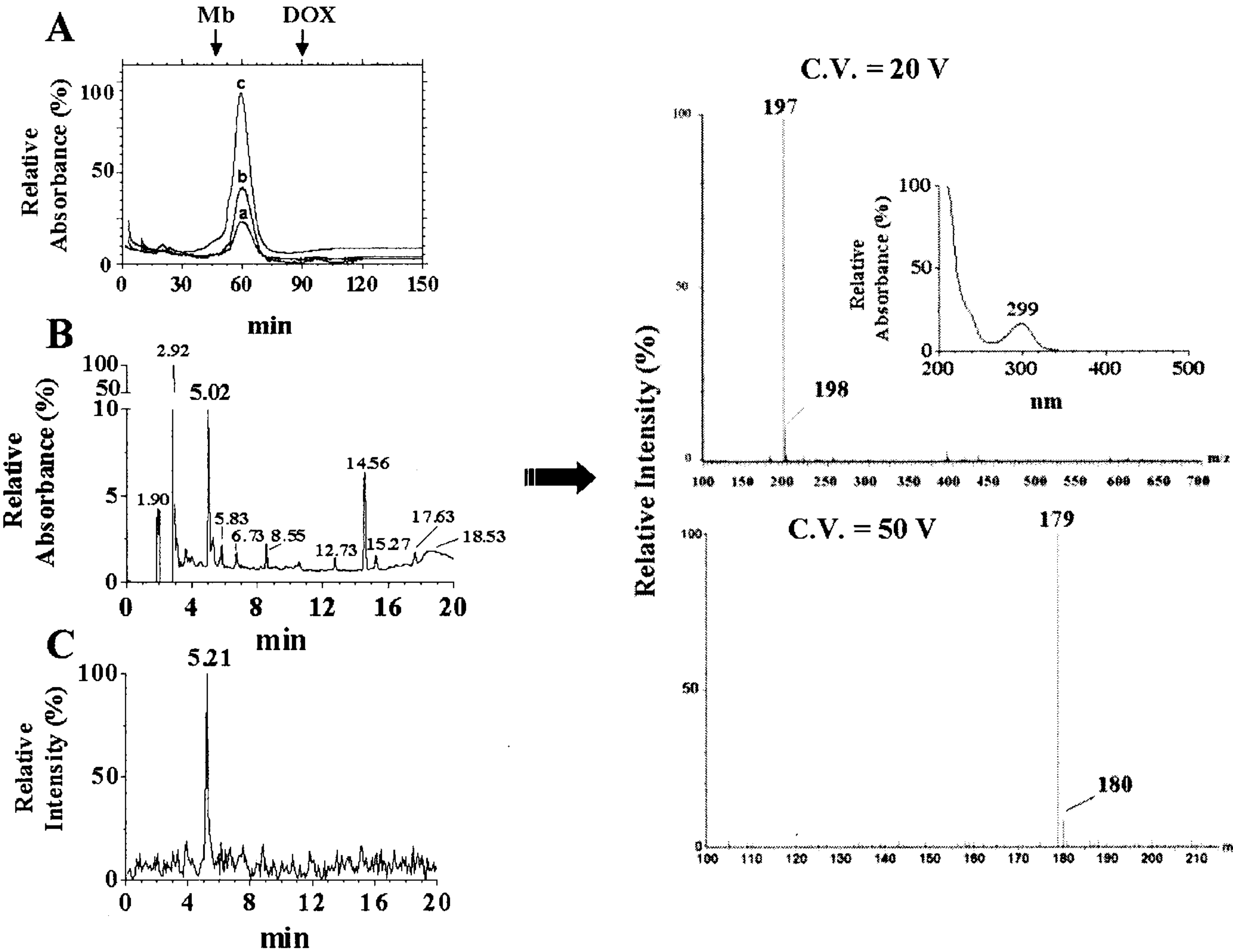

FIG. 5. **Gel permeation, HPLC, and HPLC/ESI(+)-MS of DOX/Mb^{IV} ultrafiltrates.** Incubations (1 ml final volume) contained DOX (100–500 μM) and equimolar Mb^{III}, always reacted with a 2-fold excess of H_2O_2. After 10 min, reactions mixtures were added with catalase (2600 units) and ultrafiltered. *A* shows gel permeation of ultrafiltrates derived from Mb^{IV}-degraded DOX (100 μM (*line a*), 200 μM (*line b*), or 500 μM (*line c*)); the *upper arrows* indicate the retention times of Mb^{III} or DOX standards chromatographed under comparable conditions. *B* shows HPLC of gel-filtered fraction c in *A*, and *C* shows the corresponding HPLC/ESI(+)-MS chromatogram. ESI(+)-MS of the HPLC fraction eluted at 5.21 min was carried out at C.V. = 20 or 50 V; the *inset* in the *upper panel* shows the UV spectrum associated with the HPLC fraction eluted at 5.02 min.

more sensitive and convenient than the positive mode for performing these experiments. Fig. 7*A* shows the TIC chromatogram of authentic 3-methoxyphthalic acid, characterized by a peak at 9.6 min, and Fig. 7*B* shows the product ion scan spectrum corresponding to that peak. The spectrum consisted of an ion at *m/z* = 195, corresponding to the deprotonated molecule $[M - H]^-$ of 3-methoxyphthalic acid in the ESI(−) mode, and two more ions at *m/z* = 151 and 107. The latter probably indicated, respectively, the loss of one $[M - H - 44]^-$ or both $[M - H - 88]^-$ carboxyl groups of 3-methoxyphthalic acid (see also Fig. 7*B*, *inset*). The ultrafiltrates of DOX/Mb^{IV} incubations gave the same TIC chromatogram of 3-methoxyphthalic acid (Fig. 7*C*) as well as the same MS/MS fragmentation pattern with ions at *m/z* = 195, 151, and 107 (Fig. 7*D*).

Stoichiometries and Time Courses—HPLC analyses of DOX/Mb^{III}/H_2O_2 incubations showed that 3-methoxyphthalic acid formation closely paralleled DOX degradation induced by increasing levels of Mb^{IV} (Fig. 8*A*).

Doxorubicin degradation and 3-methoxyphthalic acid formation occurred in a time-dependent manner (Fig. 8*B*), but the stoichiometry of 3-methoxyphthalic acid formation *versus* DOX degradation decreased from ~0.5 to ≤ 0.12 in 3–4 min (Fig. 8*C*). This was not attributable to DOX depletion or secondary metabolization of 3-methoxyphthalic; in fact, ~60–70% of DOX was still available for further degradation after 3–4 min reaction between Mb^{III} and H_2O_2, and 3-methoxyphthalic acid was stable to prolonged incubation with Mb^{III} or H_2O_2 or Mb^{III}/H_2O_2 (*cf.* Fig. 8, *B* and *C,* and *inset*). Data analysis showed that the stoichiometry decreased due to an ~4-fold increase of DOX degradation that was not accompanied by an increased formation of 3-methoxyphthalic acid. Because these changes occurred 3–4 min after mixing Mb^{III} with H_2O_2, which is the time when the anthracycline began reacting with Fe^{IV}=O, we considered that compound II was highly effective at oxidizing DOX but generated several product(s) other than 3-methoxyphthalic acid, eventually decreasing the stoichiometry of 3-methoxyphthalic acid formation *versus* DOX degradation. This possibility was anticipated by the fact that hemin/H_2O_2 (a surrogate of compound I) allowed DOX oxidation and 3-methoxyphthalic acid formation to proceed stoichiometrically coupled for several more minutes than did Mb^{III}/H_2O_2 (a source of both compound I and II) (not shown). Further evidence was obtained by adding DOX 1 h after mixing Mb^{III} with H_2O_2, a time when both porphyrin and globin radicals had decayed (2, 32, 33), whereas the long lived Fe^{IV}=O was still present at ~50% its initial absorbance (Fig. 9*A*). Under these defined conditions, the ad-

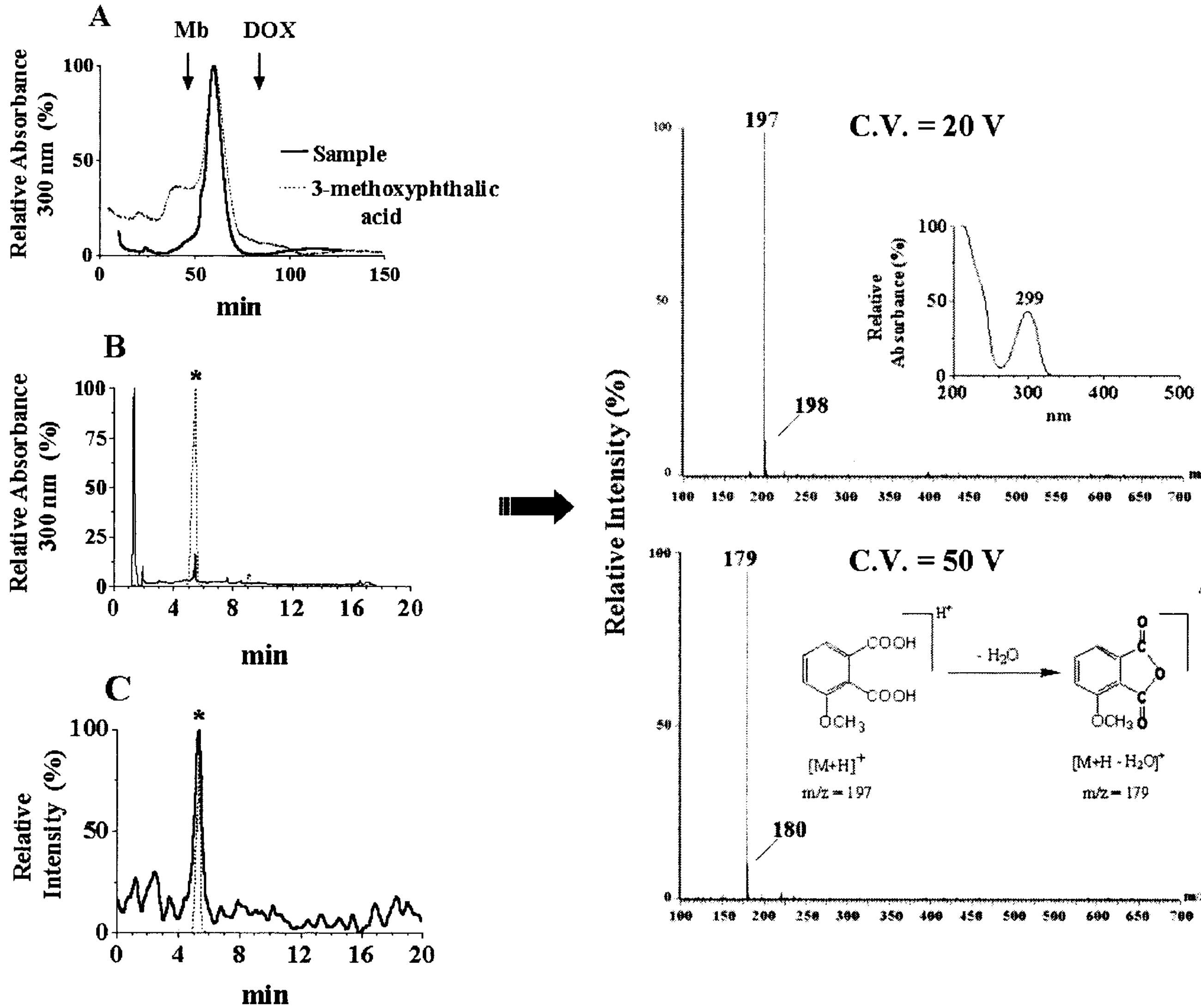

FIG. 6. **3-Methoxyphthalic acid, co-chromatography with ultrafiltrates of Mb^{IV}-degraded DOX and ESI(+)-MS analysis.** *A–C* show co-chromatography of 3-methoxyphthalic acid (*dotted lines*) with Mb^{IV}-degraded DOX (*solid lines*) in gel permeation, HPLC-UV, and HPLC/ESI(+)-MS. Experimental conditions were the same as in Fig. 5. *A* and *B,* absorbances were monitored at 300 nm. ESI(+)-MS of 3-methoxyphthalic acid was carried out at C.V. = 20 or 50 V. The *inset* in the *upper panel* shows the UV spectrum of HPLC-eluted 3-methoxyphthalic acid; the *inset* in the *lower panel* shows fragmentation of the quasi-molecular ion $[M + H]^+$ at *m/z* 197 to its possible protonated anhydride $[M + H - 18]^+$ at *m/z* 179.

dition of DOX resulted in a very rapid (~1 min) and complete disappearance of residual $Fe^{IV}{=}O$, coupled with regeneration of the spectrum of Mb^{III} and extensive degradation of the anthracycline; however, the yield of 3-methoxyphthalic acid was very low, according to a stoichiometry of 3-methoxyphthalic acid formation *versus* DOX degradation of less than ~0.02 (Fig. 9, *A* and *B*). Finally, the apparent k_{cat}/k_m value of H_2O_2/Mb^{III} for formation of 3-methoxyphthalic acid was 2 orders of magnitude lower than that determined for DOX degradation ($1.5 \pm 0.2 \times 10$ *versus* $1.8 \pm 0.2 \times 10^3$ M^{-1} s^{-1}; $n = 3$), which was consistent with the fact that both compound I and compound II were involved in oxidizing DOX but compound II uncoupled anthracycline degradation from formation of 3-methoxyphthalic acid.

Studies of Other Anthracyclines—Doxorubicin was compared with other approved or investigational anthracyclines like DNR and IDA or naphthazarin. The only difference between DOX and DNR is that the side chain of DOX terminates with a primary alcohol, whereas that of DNR terminates with a methyl (*cf.* Fig. 1). This difference did not alter the usual pattern of Mb^{IV}-anthracycline reactions, as evidenced by DNR degradation and formation of essentially the same amounts of 3-methoxyphthalic acid observed with DOX at low or high Mb^{IV}:anthracycline ratios (Table I). Idarubicin (4-demethoxy-DNR) was similarly susceptible to degradation but released phthalic acid in place of 3-methoxyphthalic acid due to the absence of a methoxy residue in ring D. Naphthazarin, an investigational model compound reproducing only the quinone-hydroquinone rings of DOX, underwent degradation but obviously released no phthalate due to the absence of ring D (see also Fig. 1 and Table I); attempts to identify the products of naphthazarin degradation by ESI-MS were unsuccessful. Comparisons between DOX and DNR or IDA therefore showed that 3-methoxyphthalic acid and simple phthalic acid were common products of Mb^{IV}-dependent degradation of anthracyclines with methoxy or demethoxy ring D. On the other hand, results obtained with naphthazarin showed that anthracycline degradation occurred in the central quinone-hydroquinone portion of these molecules.

3-Methoxyphthalic Acid Formation in Vivo—Mice were treated with DOX intravenously, and their organs were analyzed after 4 or 24 h (Table II). At 4 h both the heart and liver and kidneys contained sizeable amounts of DOX or

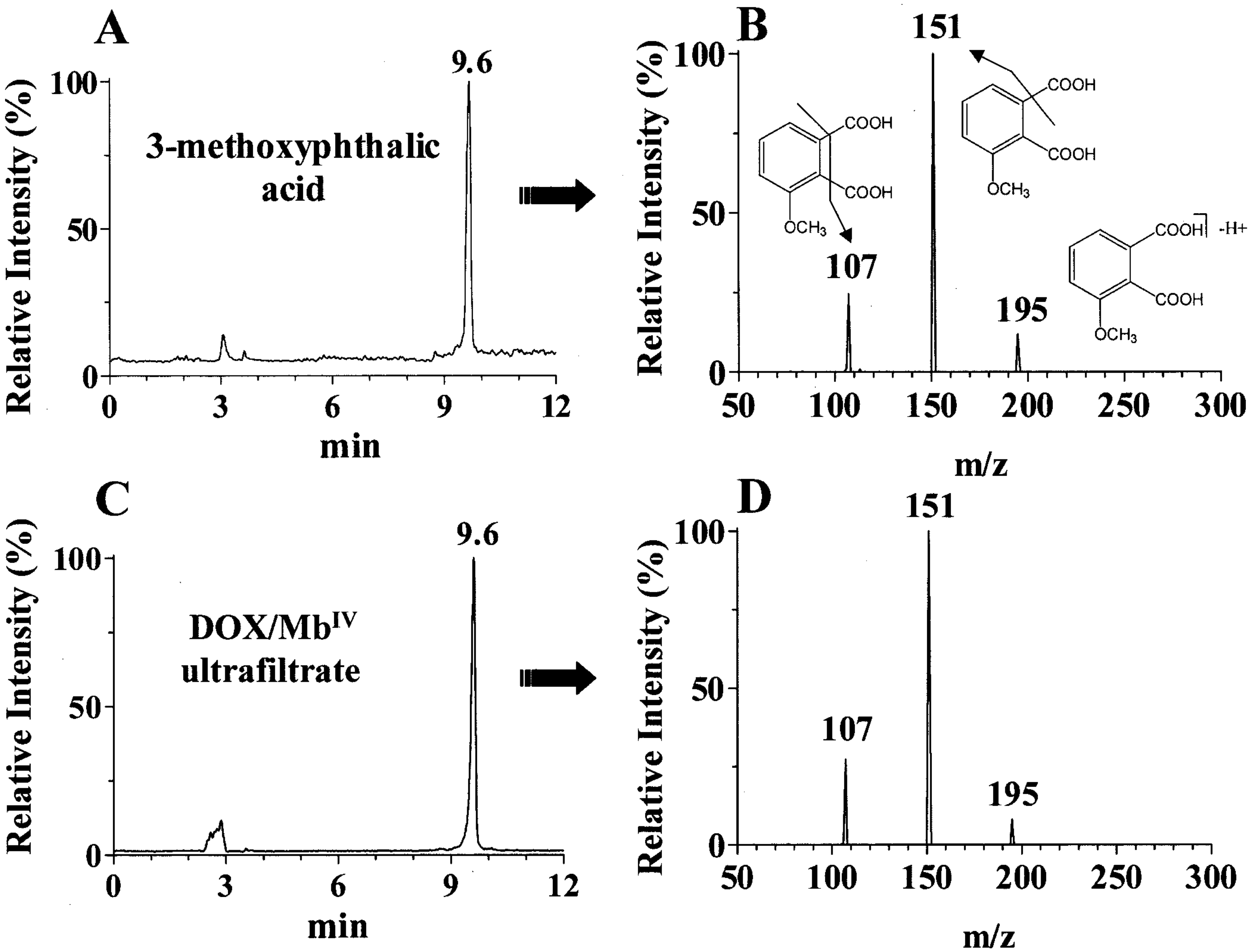

FIG. 7. **LC-ESI(−) MS/MS of authentic 3-methoxyphthalic acid and DOX/Mb^{IV} ultrafiltrates.** All conditions were as described under "Experimental Procedures." *A* shows the TIC chromatogram of authentic 3-methoxyphthalic acid, with a peak at 9.6 min, and *B* shows the product ion scan spectrum corresponding to that peak. The fragmentation pattern (*inset*) probably indicates the loss of one or both carboxyl groups. *C* and *D* show that similar results were obtained with an ultrafiltrate derived from DOX/Mb^{IV} incubations.

metabolites (like the C-13 secondary alcohol metabolite and hydroxy- or deoxyaglycones), which retained the same fluorescence of unchanged DOX due to the presence of an intact tetracyclic ring in their molecule. The values of DOX and/or fluorescent metabolites decreased, to a variable extent, at 24 h due to a tissue clearance of both parent drug and its metabolites. Different results were obtained in regard to 3-methoxyphthalic acid. As also shown in Table II, 3-methoxyphthalic acid was found in the heart at 4 h and increased further at 24 h, reaching levels that were higher than those of DOX and fluorescent metabolites. The liver contained 3-methoxyphthalic acid only at 24 h, but its levels were only ~30% of those found in the heart at the same post-treatment time. Kidneys did not contain 3-methoxyphthalic acid at either 4 or 24 h. These results showed that (i) 3-methoxyphthalic acid was formed *in vivo* after DOX administration; (ii) the heart was more active than other organs in degrading DOX to 3-methoxyphthalic acid; and (iii) there were conditions when the cardiac levels of 3-methoxyphthalic acid exceeded those of unchanged DOX or its metabolites.

3-Methoxyphthalic Acid Formation in Human Myocardium—Human myocardial strips were exposed to 1 or 10 μM DOX, concentrations found in the plasma of patients after slow or bolus infusions of the drug (34). At the end of the experiments the strips contained very low amounts of residual unchanged DOX but contained sizeable amounts of fluorescent metabolites and 3-methoxyphthalic acid. At 1 μM DOX intramyocardial levels of 3-methoxyphthalic acid were higher than those of fluorescent metabolites; at 10 μM DOX the levels of fluorescent metabolites increased and exceeded those of 3-methoxyphthalic acid, but the latter remained several times higher than unchanged DOX (Table III). By having demonstrated that 3-methoxyphthalic was an important component of the metabolic fate of DOX in human myocardium, we performed experiments to confirm that it was formed by H_2O_2-activated myoglobin. Three lines of evidence showed that this was the case. First, incubation of a whole homogenate of human myocardium with DOX and H_2O_2 resulted in anthracycline degradation and 3-methoxyphthalic acid formation, but these processes became more evident when H_2O_2 was added to cytosol, *i.e.* the subcellular fraction containing myoglobin. Second, treatment of cytosol with 65% ammonium sulfate removed ~80% of myoglobin by salting out and gave an Mb$^-$ cytosol that was essentially inactive at degrading DOX or generating 3-methoxyphthalic acid upon incubation with H_2O_2. Finally, Mb$^-$ cytosol regained activity in DOX degradation and 3-methoxyphthalic acid formation after reconstitution with its salted out myoglobin (see Fig. 10, *A–D,* and legend).

Studies with Isolated Cardiomyocytes—We have shown previously (25) that the embryonic rat heart-derived cell line H9c2

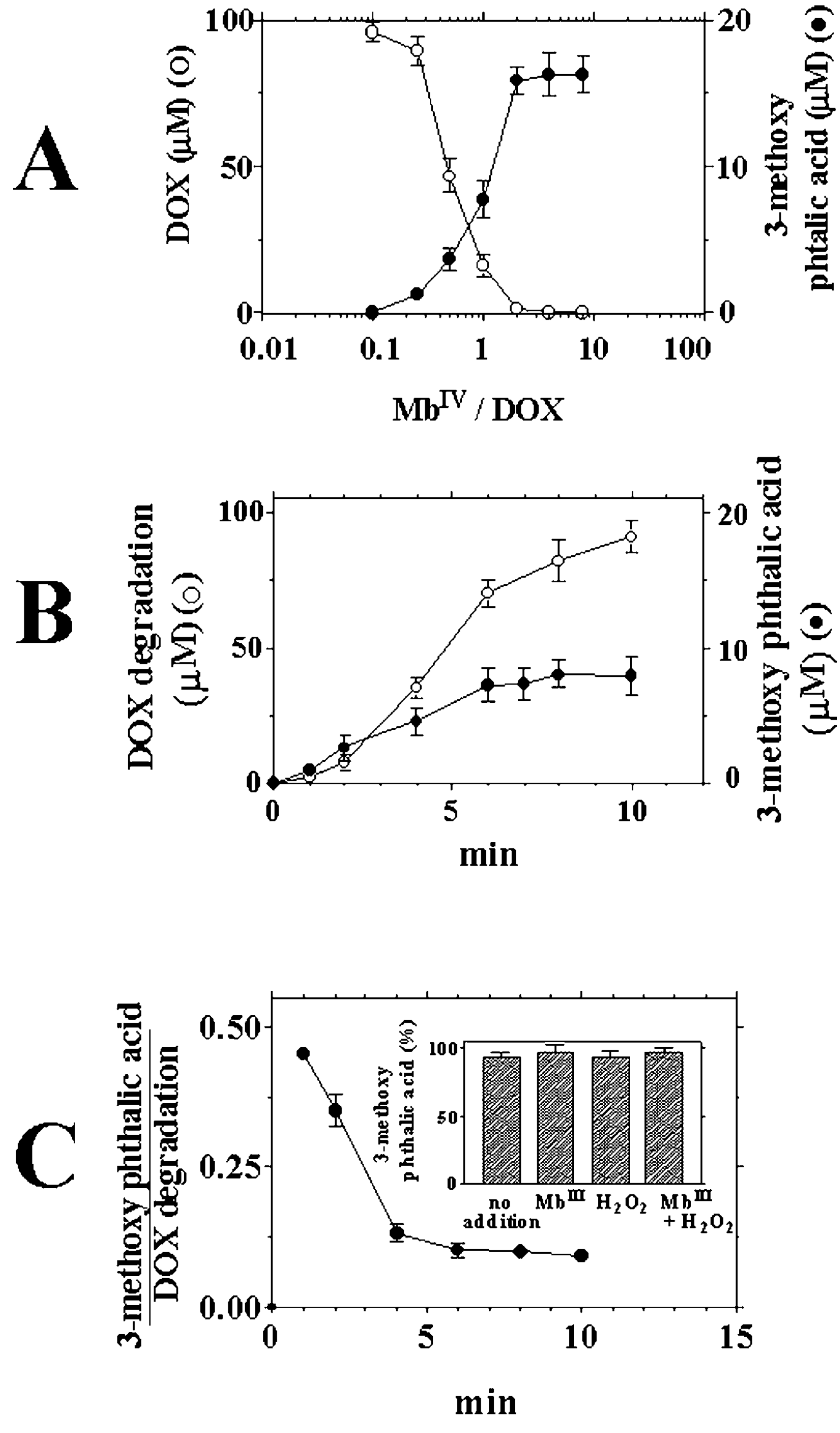

FIG. 8. **Stoichiometries and time courses of DOX degradation and 3-methoxyphthalic acid formation.** *A*, increasing Mb^{IV}:DOX ratios were obtained by incubating 100 μM DOX with 10 μM to 0.8 mM Mb^{III}, always reacted with a 2-fold excess of H_2O_2. After 10 min DOX and 3-methoxyphthalic acid were measured by HPLC as described under "Experimental Procedures." *B*, DOX and Mb^{III} (both 100 μM) were incubated with 200 μM H_2O_2. Reaction mixtures were analyzed at regular times for DOX degradation (initial DOX-residual DOX) and 3-methoxy phthalic acid formation. *C* shows time-dependent decrease of the stoichiometry of 3-methoxyphthalic acid *versus* DOX degradation. Values were determined based on data in *B*; the *inset* shows the recovery of 3-methoxyphthalic acid (100 μM) after 10 min of incubation with equimolar Mb^{III} and/or 200 μM H_2O_2. All values were means ± S.E. of three experiments.

offers a convenient and reproducible model for evaluating cardiotoxicity induced by DOX or related compounds *in vitro*. Therefore, H9c2 cardiomyocytes were exposed to 0.01–10 μM 3-methoxyphthalic acid to see whether it caused increased or decreased toxicity compared with equimolar DOX. Inasmuch as cardiac tissue has a density very similar to that of water (1 g/ml) (14), the range of concentrations of 3-methoxyphthalic acid in the incubation medium was broad enough to include and exceed those detected in mouse heart (1–3 nmol/g, *cf.* Table II) or in human myocardial strips (0.37–0.44 nmol/g, *cf.* Table III). As shown in Fig. 11, 3-methoxyphthalic acid never reduced cardiomyocyte viability in the MTT assay; in contrast, a significant loss of viability occurred if cardiomyocytes were exposed to ≥1 μM DOX. Thus, 3-methoxyphthalic acid was essentially non-toxic compared with DOX.

DISCUSSION

We have shown that Mb^{IV}, a pseudoperoxidase, is more effective than authentic peroxidases at promoting the oxidative degradation of DOX, exhibiting a k_{cat}/k_m 1 order of magnitude higher than that of HRP or LPO. Time course analyses of $Fe^{IV}=O$ formation and decay *versus* DOX degradation, and experiments conducted with hemin/H_2O_2 as a source of porphyrin radical/$Fe^{IV}=O$, indicate that DOX is oxidized by the porphyrin radical of a compound I-like species and then by the $Fe^{IV}=O$ moiety of a compound II-like species (*cf.* Figs. 2–4).

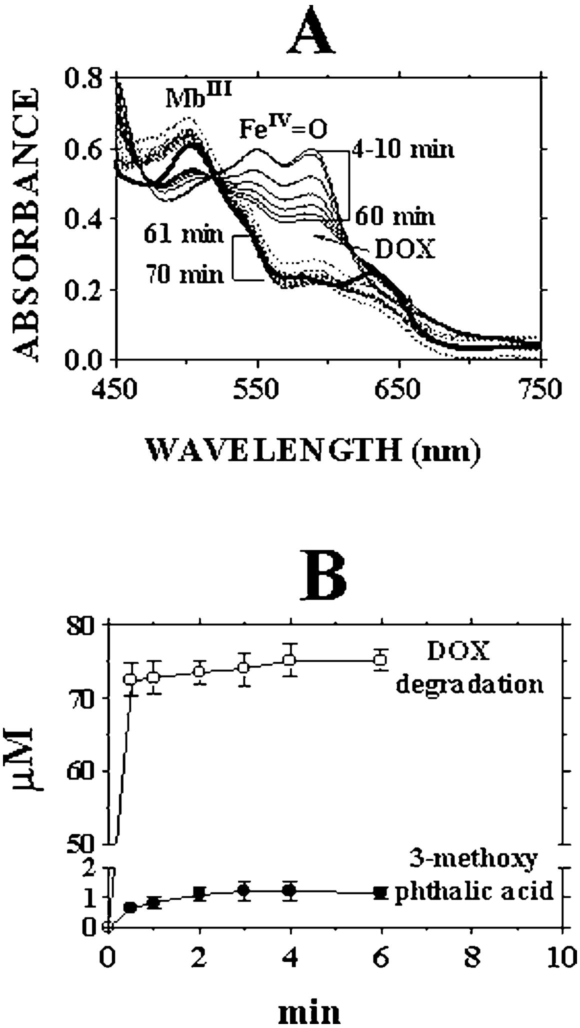

FIG. 9. **DOX degradation and 3-methoxyphthalic acid formation.** Results were obtained by adding DOX 1 h after mixing Mb^{III} with H_2O_2. *A*, Mb^{III} (50 μM) was reacted with H_2O_2 (100 μM) to form Mb^{IV}/Fe^{IV}=O (peaks at 546 and 586 nm). Spectra were taken at 4 and 10 min and then every 10 min until 60 min (*solid lines*). At 60 min DOX (50 μM) was added, and spectra were taken every min for 10 consecutive min (*dotted lines*). The *boldface trace* is the spectrum of unreacted Mb^{III}. *B*, experimental conditions were as in *A*, except that Mb^{III} and H_2O_2 were 100 and 200 μM, respectively. After 60 min, DOX (100 μM) was added, and aliquots were taken every min and assayed for DOX degradation (initial DOX − residual DOX) or 3-methoxyphthalic acid formation. Values were means ± S.E. of three determinations.

The ease with which the porphyrin radical of compound I dissipates in the globin clearly calls attention also on a possible involvement of amino acid radicals centered at Tyr^{103} or Trp^{14}, for example (4–7). In this regard we have data showing that tryptophan radicals, generated by oxidizing tryptophan with HRP, lack reactivity toward DOX; tyrosine radicals, generated through the same procedure, did degrade DOX but acetylation of myoglobin tyrosine residues did not decrease the k_{cat}/k_m of Mb^{III}/H_2O_2 for degradation of DOX (not shown). These reasonings do not completely rule out a possible role for globin-centered reactive species in oxidizing DOX. Free radicals are known to transfer from one site of the globin to another, and chemical modifications or site-directed mutagenesis of the primary sites of free radical formation may not prevent the formation of other radicals on nearby residues (32). Moreover, Mb^{IV} may display an additional radical signal that is not centered at Tyr or Trp and has been tentatively assigned to oxidation of an unidentified aromatic amino acid in close proximity to the heme pocket (32). The possible role of globin radicals as additional determinants of DOX oxidation therefore remains a matter of consideration.

TABLE I
Anthracycline degradation and phthalic acids formation, comparisons between DOX and analogs

Drugs were 100 μM, and Mb^{IV}:drug ratios (0.5/2:1) were obtained by incubation with 50-200 μM Mb^{III} always reacted with 2-fold H_2O_2. After 10 min, incubations were assayed by HPLC for drug degradation (initial-residual) and phthalates formation. Values were means ± S.E. of three experiments. —, not detectable.

Drug	Mb^{IV}:drug	Degradation	3-Methoxyphthalic acid	Phthalic acid
		μM		
DOX	0.5:1	53 ± 7	3.8 ± 0.8	—
	2:1	98 ± 2	16 ± 1	—
DNR	0.5:1	40 ± 4	3.6 ± 0.7	—
	2:1	98 ± 1	13 ± 1.5	—
IDA	0.5:1	62 ± 9	—	14 ± 1.5
	2:1	99 ± 1	—	23 ± 2
Naphthazarin	0.5:1	83 ± 20	—	—
	2:1	97 ± 2	—	—

TABLE II
Levels of DOX, fluorescent metabolites, and 3-methoxyphthalic acid in organs of mice treated with DOX

Other conditions are as described under "Experimental Procedures." —, not detectable.

	DOX	Fluorescent metabolites[a]	3-Methoxyphthalic acid
		nmol/g	
Heart			
4 h	8.1 ± 0.5	0.3 ± 0.05	1.7 ± 0.1
24 h	1.9 ± 0.2[b]	0.2 ± 0.1	3.0 ± 0.3[c]
Liver			
4 h	6.1 ± 0.8	0.4 ± 0.1	—
24 h	1.3 ± 0.2[b]	0.1 ± 0.02[d]	1.0 ± 0.2
Kidney			
4 h	12.6 ± 1	1.9 ± 0.3	—
24 h	9.1 ± 0.8[e]	0.2 ± 0.1[f]	—

[a] Sum of C-13 secondary alcohol metabolite and hydroxy- or deoxy-aglycones. Values were means ± S.E. (n = 9).
[b] $p < 0.01$ or < 0.05 *versus* 4 h.
[c] $p < 0.025$ *versus* 4 h and $p < 0.05$ *versus* DOX 24 h.
[d] $p < 0.01$ versus 4 h.
[e] $p < 0.05$ *versus* 4 h.
[f] $p < 0.01$ *versus* 4 h.

TABLE III
Levels of DOX and fluorescent metabolites versus 3-methoxyphthalic in human myocardial strips exposed to 1 or 10 μM DOX

DOX	Unchanged DOX	Fluorescent metabolites[a]	3-Methoxyphthalic acid
μM		*nmol/g*	
1	0.02 ± 0.01	0.27 ± 0.04	0.44 ± 0.06
10	0.04 ± 0.01	1.2 ± 0.2[b]	0.37 ± 0.05

[a] Sum of C-13 secondary alcohol metabolite and hydroxy- or deoxyaglycones. Other conditions were as described under "Experimental Procedures."
[b] Values were means of ± SE (n = 4). $p < 0.01$ *versus* 1 μM.

Chromatographic, UV, and mass spectrometry analyses provide novel evidence that a biologic oxidant like Mb^{IV} degrades DOX to 3-methoxyphthalic acid, product of oxidative modifications of the methoxy-substituted ring D (*cf.* Figs. 1 and 5–7). Biochemical evidence for the formation of oxidized ring D products was also offered by the fact that methoxy or demethoxy analogs, like DNR or IDA, similarly released 3-methoxyphthalic acid or simple phthalic acid after exposure to Mb^{IV} (*cf.* Table I). Previously, the formation of 3-methoxyphthalic acid or other products of ring D oxidation (like *e.g.* 3-methoxysalycilic acid) was only observed under artificial conditions such as permanganate oxidation of investigational anthraquinones (35) or riboflavin-mediated photooxidation of DOX (36).

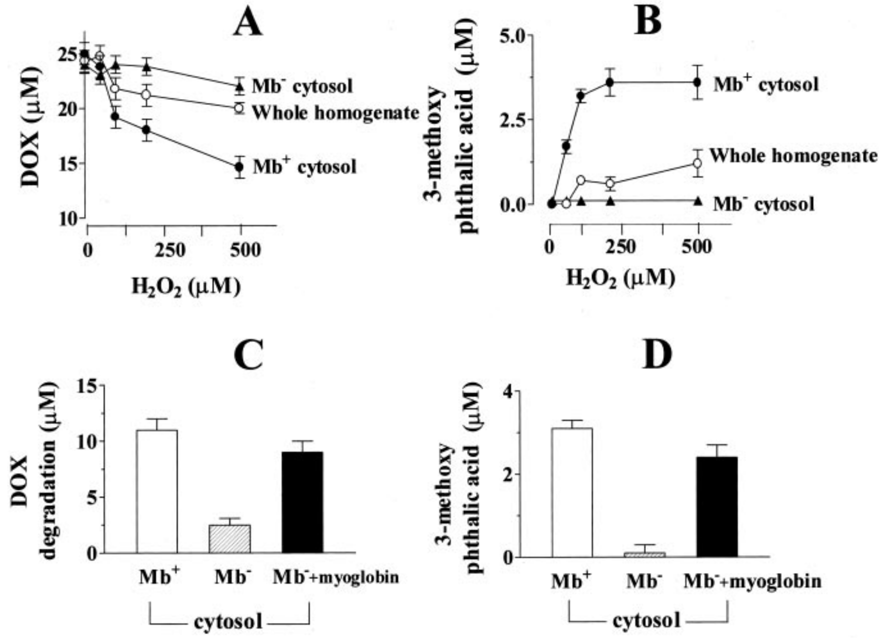

FIG. 10. **DOX degradation and 3-methoxyphthalic acid formation in human myocardium; role of myoglobin.** Incubations (200 μl final volume) contained 15 μM DOX and 3 mg of protein/ml of human heart whole homogenate (1.3 nmol of Mb^{III}/mg of protein), Mb^+ cytosol (2.9 nmol of Mb^{III}/mg of protein), or Mb^- cytosol (0.6 nmol of Mb^{III}/mg of protein). Reactions were started by adding increasing amounts of H_2O_2, as indicated. After 10 min incubations were assayed for DOX (*A*) or 3-methoxyphthalic acid (*B*). *C* and *D*, DOX degradation (initial DOX-residual DOX) and 3-methoxyphthalic acid formation were measured in incubations containing 500 μM H_2O_2 and 3 mg of protein/ml of Mb^+ cytosol, Mb^- cytosol, or Mb^- cytosol reconstituted with its salted out myoglobin (2.3 nmol of Mb^{III}/mg of protein, dialyzed just prior to experiments to remove ammonium sulfate). Values were means ± S.E. of triplicate experiments.

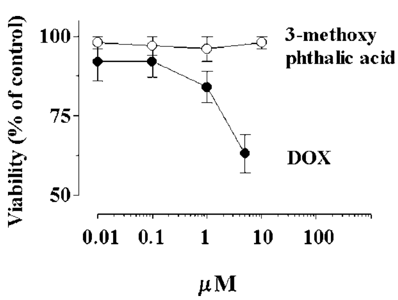

FIG. 11. **Toxicity of DOX or 3-methoxyphthalic acid to H9c2 cardiomyocytes.** Toxicity was evaluated as the percentage of viable cells (MTT assay) after an overnight exposure to DOX or 3-methoxyphthalic acid. Values were means ± S.E. of four separate determinations.

The complete sequence of events leading to the formation of 3-methoxyphthalic acid cannot be envisaged at this time as Mb^{IV}-DOX interactions released other products that were detected by HPLC but could not be characterized by ESI(+) MS (*cf.* Fig. 5, *B* and *C*).[3] The formation of such unknown products seemed to occur primarily during oxidation of DOX by compound II, characterized by a stoichiometry of 3-methoxyphthalic acid formation *versus* DOX degradation much lower than that determined for oxidation of DOX by compound I (*cf.* Fig. 8*C*). Nevertheless, there are at least two mechanisms that may help to anticipate how DOX releases 3-methoxyphthalic acid after oxidation by Mb^{IV}. As mentioned, anthracycline degradation by Mb^{IV} or peroxidases is preceded by one-electron oxidation of the hydroquinone in juxtaposition to the quinone (14, 16). This reaction generates a semiquinone whose disproportionation regenerates a hydroquinone while also forming a diquinone (16). Because diquinones are rather unstable compounds that decompose spontaneously (16), oxidation of DOX from its hydroquinone-quinone form to a diquinone form may represent a plausible mechanism to explain how Mb^{IV} promotes opening and degradation of the planar anthracycline ring. In keeping with this concept, we found that also naphthazarin, a two-ring membered compound reproducing the quinone-hydroquinone portion of anthracyclines, was degraded by Mb^{IV} (*cf.* Table I); in contrast, one ring-membered hydroquinones like simple hydroquinone or 2,5-di-*tert*-butylhydroquinone were not degraded by Mb^{IV} but converted quantitatively to the corresponding stable quinones (not shown). Another mechanism to be taken into account when considering the carboxylation of ring D in the form of 3-methoxyphthalic acid may be inferred from the ability of lignin peroxidase to oxidatively degrade 3,4-dimethoxybenzyl methyl ether to muconic acid (37). These reactions suggest that peroxidases can cleave aromatic rings and that secondary reactions, mediated by oxygen activation/addition, can result in formation of carboxylic acid derivatives (37).

Doxorubicin has long been known to undergo one-electron reduction of its quinone, two-electron reduction of its side chain C-13 carbonyl group, or reductive cleavage of the glycosidic bond linking its tetracyclic ring with an amino sugar (*cf.* Fig. 1). While forming semiquinone free radicals and secondary alcohol or deoxyaglycone derivatives that have been implicated to explain, at least in part, the cardiotoxic properties of DOX (25, 38, 39), these reductive processes do not induce any important or irreversible loss of the optical and fluorescent properties of unchanged DOX. However, the administration of DOX to humans and laboratory animals was shown to generate also nonfluorescent compounds that were tentatively attributed to oxidative degradation rather than reductive biotransformation of the drug (40, 41). Our results indicate that 3-methoxyphthalic acid is formed not only in reconstituted chemical systems but also in the heart of mice treated with DOX or in human myocardial biopsies exposed to concentrations of DOX similar to those found in the plasma of patients (*cf.* Tables II and III). In mice, the heart generates considerably more 3-methoxyphthalic acid than organs like liver or kidneys, as one would expect if Mb^{IV} were more effective than other peroxidases at degrading DOX. Comparisons between human heart homogenate and Mb^+ or Mb^- cytosol also confirmed that cytosol served a preferred site of DOX degradation and 3-methoxyphthalic acid formation, and that both processes could be abolished or reactivated by removing or reintroducing myoglobin (*cf.* Fig. 10). Our results therefore identify Mb^{IV} and 3-methoxyphthalic acid among the long sought catalyst(s) and product(s) of anthracycline oxidation in biologic systems.

[3] In re-attempting characterization of other products of DOX degradation products by ESI(−)-MS, we occasionally detected ions at *m/z* = 203 or 237, 242, and 265. However, further characterizations and unambiguous assignment of these ions were not possible.

Experiments with DOX-treated mice or human myocardial strips exposed to DOX show that there may be conditions when the cardiac levels of 3-methoxyphthalic acid exceed those of unchanged DOX or fluorescent metabolites (*cf.* the heart of mice 24 h after DOX administration or myocardial strips exposed to 1 μM DOX) (*cf.* Tables II and III). These observations suggest that 3-methoxyphthalic acid might be an important determinant of the mode of action of anthracyclines and offered a rationale to see how it compared with DOX in inducing toxicity to cardiomyocytes. We demonstrate that concentrations of 3-methoxyphthalic acid equal to or several times higher than those found in whole cardiac tissues lack toxicity to isolated cardiomyocytes; under comparable conditions, however, low concentrations of DOX are highly toxic to cardiomyocytes (*cf*. Fig. 11). Although the biologic action(s) of other currently unknown degradation products cannot be disregarded, these results raise the possibility that the pseudoperoxidase activity of Mb^{IV} may serve an important mechanism to diminish cellular levels of anthracyclines and to divert them from formation of toxic reduced metabolites toward formation of non-toxic oxidized products.

In summary, we have shown the following. (i) Mb^{IV} is a very good catalyst of anthracycline degradation. (ii) 3-Methoxyphthalic acid and simple phthalic acid are common products of the oxidative degradation of methoxy-substituted or demethoxy analogs like DOX and DNR or IDA, respectively. (iii) The compounds I and II of Mb^{IV} are sequentially involved in oxidizing DOX, although with different stoichiometries of 3-methoxyphthalic acid formation *versus* anthracycline degradation. (iv) 3-Methoxyphthalic acid is an abundant product of DOX degradation in the heart of laboratory animals or in human myocardium. (v) 3-Methoxyphthalic acid does not induce toxicity to cardiomyocytes. These results unravel novel functions for Mb^{IV} and pose mechanism-based foundations to see whether reactions of Mb^{IV} with anthracyclines may be exploited to improve cardiac tolerability of these otherwise useful agents.

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