Inactivation of Anthracyclines by Serum Heme Proteins

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We have previously shown that the anticancer agent doxorubicin undergoes oxidation and inactivation when exposed to myeloperoxidase-containing human leukemia HL-60 cells, or to isolated myeloperoxidase, in the presence of hydrogen peroxide and nitrite. In the current study we report that commercial fetal bovine serum (FBS) alone oxidizes doxorubicin in the presence of hydrogen peroxide and that nitrite accelerates this oxidation. The efficacy of inactivation was dependent on the concentration of serum present; no reaction was observed when hydrogen peroxide or serum was omitted. Peroxidase activity assays, based on oxidation of 3,3',5,5'-tetramethylbenzidine, confirmed the presence of a peroxidase in the sera from several suppliers. The peroxidative activity was contained in the >10000 MW fraction. We also found that hemoglobin, a heme protein likely to be present in commercial FBS, is capable of oxidizing doxorubicin in the presence of hydrogen peroxide and that nitrite further stimulates the reaction. In contrast to intact doxorubicin, the serum + hydrogen peroxide + nitrite treated drug appeared to be nontoxic for PC3 human prostate cancer cells. Together, this study shows that (pseudo)peroxidases present in sera catalyze oxidation of doxorubicin by hydrogen peroxide and that this diminishes the tumoricidal activity of the anthracycline, at least in in vitro settings. Finally, this study also points out that addition of H₂O₂ to media containing FBS will stimulate peroxidase-type of reactions, which may affect cytotoxic properties of studied compounds.

Introduction

Peroxidases are heme proteins that utilize H₂O₂ to convert substrates to reactive metabolites. We have previously reported that human promyelocytic leukemia HL-60 cells, which are rich in MPO, as well as isolated MPO and LPO enzymes, catalyze oxidation of anthracycline anticancer drugs in the presence of H₂O₂. A facilitating cofactor in this reaction is NO₂⁻ (1, 2); acetaminophen and salicylic acid also enhance the reaction (3, 4). This peroxidase-dependent metabolism causes inactivation of the anthracyclines as evidenced by their suppressed in vitro toxicity in human leukemia HL-60 cells, human prostate cancer PC3 cells, and rat cardiac H9c2 myocytes (2, 5). This inactivation is due to the oxidation-initiated degradation of anthracyclines to 3-methoxysalicylic acid (3MePA) and 3-methoxysalicylic acid (3MeSA) (Figure 1), which appear to be considerably less cytotoxic than the parent anthracycline (2, 5). The peroxide required for these oxidations was provided exogenously, but it is known that cancer cells themselves produce H₂O₂ (6, 7).

Figure 1. Oxidation of DOX by MPO/H₂O₂ in the presence of nitrite leads to its degradation to 3-methoxysalicylic acid (3MePA) and 3-methoxysalicylic acid (3MeSA).

Materials. DOX, NaNO₂ (99%), H₂O₂ (8.8 M), 3,3',5,5'-tetramethylbenzidine (TMB), dimethyl formamide, 3MeSA, catalase, equine hemoglobin (ferric form, metHb), methimazole,
aminobenzoic acid hydrazide (ABAH), and azide were obtained from Sigma Chemical Company (St. Louis, MO). 3MePA was synthesized as described (2). All other chemicals were of the highest purity available. Fetal bovine sera were obtained from diverse commercial suppliers. According to manufacturers’ specifications, all FBS contain trace amounts of hemoglobin (<25 mg%).

Cell Culture. Human prostate cancer cell line PC3 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in minimum essential media supplemented with 10% FBS, l-glutamine (1.5 mM), streptomycin (76 µg/mL) (Gibco Invitrogen, Grand Island, NY), and penicillin (76 U/mL from a 100 mM NaNO2 stock solution). Experiments had been passed less than 20 times.

Oxidation of DOX by FBS/H2O2. Oxidation of DOX was measured following changes in the drug’s characteristic absorption band at 480 nm using an Agilent diode array spectrophotometer model 8453 (Agilent Technologies, Inc., Chesterfield, MO). To DOX in RPMI 1640 media, or in phosphate buffer saline (pH 7.0), containing FBS at indicated concentrations, was added 1 mM H2O2 (freshly diluted from a 8.8 M stock) and/or 1 mM NaNO2 (10 µL/mL from a 100 mM NaNO2 stock solution). Experiments were carried out at 37 °C in a tissue culture incubator with a 5% CO2 humidified atmosphere or at room temperature (22 °C) for some experiments. At specific time points, aliquots of the samples were withdrawn and centrifuged at 16000g for 2 min. The supernatants were then read at 480 nm. The amount of drug remaining in the supernatant was calculated using ε480 = 11500 M⁻¹ cm⁻¹ for anthracyclines (12). The dependence of DOX oxidation on [H2O2] was determined by measuring the initial rate of the loss of absorbance at 480 nm over the period up to 30 min after start of the reaction (H2O2 addition) in 100% FBS and at [H2O2] ranging from 0 to 0.5 mM. For some experiments FBS was dialyzed against water for 3 days through Spectrapor 6000 (8000 MW cutoff dialysis membrane) (Spectrum Medical Ind. Inc., Rancho Dominguez, CA).

Oxidation of DOX by methHb/H2O2. To DOX (10 µM) in phosphate buffer pH 7.0 (50 mM) was added methHb (13 µM in heme) and A480 was measured every minute for 5 min, after which 5 µL of H2O2 stock solution was added to a final concentration of 0.37 mM and measurements were continued for 20 min. Similar experiments were performed in the presence of NaNO2 (0.30 mM). FerrylHb was prepared by adding H2O2 (0.37 mM) to methHb (50 µM). Decrease in absorbance at 500 and 630 nm, characteristic of methHb, was concomitant with the formation of a new band at 545 nm, which is interpreted as a redox transition from methHb to ferrylHb (13). An aliquot of DOX solution was then added to this preformed ferrylHb and absorption spectra were measured in 1 min intervals. Samples were measured in a spectrophotometric cuvette (1 cm light path) at ambient temperature during continuous stirring with a microstirrer. The initial concentration of methHb solutions was determined by measuring its absorbance at 405 and 500 nm (ε405 = 179 mM⁻¹ cm⁻¹ and ε500 = 10 mM⁻¹ cm⁻¹) (14).

Peroxidase Activity Assays. The peroxidase activity of FBS samples was assessed using the TMB oxidation assay as described in (15). Different volumes of media or sera were placed in 50 mM sodium acetate buffer (pH 5.4) to a total sample volume of 3.05 mL. Then 50 µL of a solution containing 100 mM TMB in dimethyl formamide was added to the samples and mixed. The assay was initiated by mixing 200 µL of 5.25 mM hydrogen peroxide in sodium acetate buffer to the samples. The samples were then incubated at room temperature for 3 min, after which the reaction was quenched with the addition of 100 µL (0.3 mg/mL) of catalase and 3.4 mL of ice-cold 200 mM acetic acid in water. Immediately, the samples were mixed and then centrifuged at 15000g for 5 min. The supernatants’ absorptions were then read at 655 nm. Sensitivity was sufficient to detect peroxidase activity with as little as 0.3% by volume FBS.

Peroxidase Activity in Fractioned Sera. Tissue culture grade FBS was placed into Amicon YM-10 10000 molecular weight cutoff centrifugal filter devices (Millipore Corporation, Bedford, MA) and according to manufacturer’s instructions they were centrifuged at 1800g for 3 h (5 °C). Pooled fractions were then analyzed for the ability to oxidize doxorubicin and TMB as compared to unfractionated sera.

Determination of Serum Heme Content. The heme concentration in FBS was determined using the method described in (16). First the heme was extracted from FBS samples into chloroform by vigorous shaking and then centrifuged to separate the organic phase. The absorbance of the chloroform extract was measured at specific wavelengths (388, 450, and 330 nm) and the absorption parameter A450 was calculated using the formula A450 = 2A388 - A350. The actual concentration of the heme in FBS samples was determined using this A450 value and a calibration curve prepared using known concentrations of hemin. Samples from five different suppliers were analyzed in triplicate.

Mass Spectrometry. Doxorubicin and the drug’s oxidation products were analyzed by HPLC combined with mass spectrometry and tandem mass spectrometry as described earlier (2).

Clonogenic Survival. PC3 cells previously seeded at 500–1000 cells/plate were incubated in the presence of intact or oxidized doxorubicin at 37 °C in a humidified incubator with 5% CO2 for 10 days. After this, the cells were fixed, stained, and counted to determine their clonogenic survival (18).

Results

Oxidation of DOX by Serum. When DOX was incubated in FBS in the presence of H2O2, the drug’s characteristic absorption band at 480 nm decreased, indicating its oxidation. The extent of DOX oxidation depended on time of incubation and serum and H2O2 concentrations. First we studied oxidation of DOX by measuring changes in absorbance at 480 nm as a function of time. When DOX was incubated with 10% FBS in RPMI 1640 in the presence of 1 mM H2O2 and 1 mM NaNO2, its concentration decreased by about 52% during a 60 min incubation (Figure 2A, trace d). The loss of the drug was slower when NaNO2 was omitted (Figure 2A, trace c) and no loss was observed in the presence of NaNO2 but without H2O2 (Figure 2A, trace b).

To examine the dependence of the efficacy of DOX oxidation on [FBS], the drug was incubated in PBS (50 mM, pH 7.0) containing H2O2 (1 mM) and NaNO2 (1 mM) in the presence of increasing proportions of FBS. Figure 2B shows changes in DOX concentrations following 1 h incubation. When both H2O2 and NaNO2 were present, the largest loss of DOX, Δ[DOX] ~ 20 µM or ~89% decrease, was observed when about 15% serum had been added, and no further decrease in the drug concentration occurred at higher FBS content (Figure 2B, trace d). When NaNO2 was omitted, the oxidation occurred at a slower rate and did not level off until about 40% serum was present (Figure 2B, trace c). There was no apparent oxidation of DOX in the absence of H2O2 whether NaNO2 was present or not, even at the highest FBS content used (Figure 2B, traces a and b).

The rate of DOX oxidation was dependent on [H2O2]. In the [H2O2] range of 25–500 µM, the initial rate of DOX oxidation increased linearly with [H2O2] (Figure 3).

Peroxidase Activity in Serum. The above observations are similar to those made using isolated LPO and MPO enzymes (1, 2) and suggest the presence of a peroxidase in FBS preparations. The peroxidase activity of FBS was determined by measuring oxidation of TMB. Figure 4A shows that there is a linear relationship between the amount of FBS present in the sample and the amount of TMB oxidized. Typical peroxidase inhibitors methimazole, ABAH, and azide markedly quenched TMB oxidation, with ABAH being the most effective (Figure 4A, inset). Using the TMB assay, we determined peroxidase
activity in FBS from different manufacturers. Figure 4B demonstrates that there is appreciable activity in all samples studied.

Fractionation Studies. We have previously found that LPO and MPO systems oxidize anthracyclines only in the presence of a catalytic cofactor, a good peroxidase substrate, such as nitrite, acetaminophen, or salicylic acid (1-3). In contrast, hemin and a low molecular weight heme peptide, microperoxidase 11, catalyzed oxidation of anthracyclines by H2O2 without any cofactors (5, 18). Also, myoglobin, a protein with pseudoperoxidase activity, supports oxidation of DOX by H2O2 alone (5, 19).

In an attempt to characterize the peroxidase found in sera, we fractionated sera using a Centricon 10000 molecular weight (MW) centrifugal filter and then assayed the fractionated sera for peroxidase activity and the ability to oxidize DOX. Using the TMB assay, it was found that the >10000 MW fraction had peroxidase activity higher by 34% compared to the unfractionated sera (not shown). Less than 5% of the activity was found in the <10000 MW fraction. These results suggest that free heme and low molecular weight heme peptides are not the major source of the peroxidative activity in FBS and points to compound(s) of MW greater than 10000, possibly heme proteins, as a carrier of this activity. When the same fractions were placed in PBS (pH 7.0) and incubated with DOX in the presence of H2O2 and NaNO2 (1 mM each), the unfractionated sera and the >10000 MW fraction caused oxidation of approximately 75% of the drug (Figure 5). The <10000 MW fraction caused virtually no change in the concentration of DOX under the same conditions (Figure 5).

We also found that FBS that was exhaustively dialyzed appeared to be more effective in supporting oxidation of DOX by H2O2 than nondialyzed FBS, both in the absence and presence of nitrite (not shown). This is most likely due to removal of low molecular weight reducing agents from FBS preparations, such as ascorbate, that could inhibit DOX oxidation.

Determination of Serum Heme Content. The peroxidative activity is most likely due to the presence of heme proteins in the sera. We determined that the amount of heme in various FBS preparations varied between 1.1 and 2.0 μM, median = 1.8 μM (n = 5). Differences in the heme content among the FBSs studied were ~20.3% and differences in the peroxidative activity were ~23.3%. Thus, there is good correlation between the heme content and the peroxidative activity.
of DOX with FBS/H2O2. In separate replicate experiments the quantity of major metabolic products generated by oxidation of DOX was then studied. Unfrac (unfractionated) is DOX incubated with 1 mM nitrite and H2O2 in FBS for 60 min. Untreated (control) is DOX alone incubated with FBS for 60 min.

Figure 6. HPLC chromatograms. Samples analyzed by LC/MS showing DOX (panel A) and its oxidation products, 3MePA and 3MeSA (panel B). DOX (100 μM) was added to FBS and was treated with 1 mM nitrite and 1 mM H2O2 for 1 h. Then catalase (100 U/mL) was added to remove residual H2O2, and the samples were fractionated with a 3000 MW cutoff centrifugal device. The filtrates were then analyzed by HPLC using authentic 3-MePA and 3-MeSA as standards. Shown are representative HPLC scans.

Quantitation of Loss of Doxorubicin and Accumulation of Metabolites. We estimated the amount of DOX lost and the quantity of major metabolic products generated by oxidation of DOX with FBS/H2O2. In separate replicate experiments the drug was exposed to FBS in the presence of H2O2 and NaN3. Oxidation of DOX (93.3 ± 3.4 μM) generated 3MePA (26.8 ± 4.4 μM) and 3MeSA (2.3 ± 1.2 μM). This indicates that 29% of the parent drug was converted to 3MePA and 3.0% to 3MeSA. By spectrophotometry, only 6.7 μM or 7.2% DOX remained after 1 h incubation. The yields were determined by HPLC based on standard curves of purified reference compounds. Figure 6 shows a typical HPLC scan of the intact and oxidized DOX. The identity of the products derived from oxidized DOX was further confirmed using mass spectrometry. Figure 7 shows MS/MS spectra of negative ions (M–H)− of m/z 167.1 (A) and 195.1 (B), attributed to 3MeSA and 3MePA, respectively. The ions of m/z 152.1 and 123.1 are from 3MeSA after loss of methyl and CO2 groups, respectively. The ion of m/z 108.1 is the ion of 152.1 after loss of CO2 group. In (B), the ion of m/z 151.1 is from 3MePA after loss of CO2 group. These data are in agreement with earlier reports in which DOX was exposed to different oxidizing systems (2, 5, 20).

Oxidation of Doxorubicin by metHemoglobin/H2O2. Because hemoglobin from ruptured erythrocytes is likely to be present in serum, and because hemoglobin possesses pseudoperoxidase activity, we examined the capacity of hemoglobin to support oxidation of DOX by H2O2. We used the commercial equine Hb that is present mostly in metHb (ferric) form. When DOX was incubated with metHb in the presence of H2O2, oxidation of the drug was apparent (Figure 8A, main panel). The inset in Figure 8A shows the corresponding time course of absorption changes at 480 nm (trace a) as a measure of DOX oxidation. Nitrite (0.33 mM) markedly accelerated DOX oxidation (Figure 8A, inset trace b). No oxidation of DOX was observed when the drug was incubated with metHb alone (initial sector of the runs) or when metHb was omitted but H2O2 was present (Figure 8A, inset trace c). This demonstrates that metHb is capable of oxidizing DOX in the presence of the peroxide alone. Figure 8A also shows spectral changes in the Hb Soret band (405 nm) during DOX oxidation in the presence of nitrite. These observations are also confirmed using human hemoglobin A0 (data not shown).

Exposure of metHb to H2O2 generates an analog of peroxidase compound I containing oxo-ferryHb (FeIV = O) and a π cation radical located on the porphyrin ring of the heme group. In the absence of an appropriate oxidizable substrate the radical site migrates from the porphyrin ring to the protein (13, 21). Reduction of this radical yields a product, which resembles peroxidase compound II. Both of these forms contain the oxo-ferry moiety known to oxidize biological substrates (21). To determine whether oxo-ferryHb is responsible for oxidation of DOX, it was prepared first by adding an excess of H2O2 (0.37 mM) to metHb (~50 μM) in pH 7.0 buffer. This caused a
We have shown that a component of FBS with a molecular mass >10000 Da, possibly a heme protein, exerts this peroxidase-like activity. According to manufacturers’ specifications, commercial FBS contains up to 25 mg% hemoglobin, which corresponds to ~3.8 μM (based on MW 64500), which is close to heme contents determined in FBSs in this study. Given the above, and considering that Hb/H2O2 is capable of oxidizing DOX, as observed in this study, it seems likely that this activity is due, at least partially, to Hb present naturally in FBS. Importantly, the Hb/H2O2 system, similar to other peroxidative systems, oxidizes NO2−, causing self-nitration as well as nitration of other proteins (22). These processes are mediated by reactive nitrogen species derived from nitrite, and the same species may play a role in oxidation of DOX (1). Thus, our observation that NO2− enhances oxidation of DOX by metHb/H2O2 is consistent with these earlier reports. We also wish to emphasize that myoglobin, a protein functionally related to Hb, has been shown to oxidize anthracyclines in the presence of H2O2 (5, 19).

**Discussion**

Our results demonstrate that FBS contains a peroxidase-like activity that can support oxidation of DOX by exogenous H2O2. The oxidation of the drug is substantial, causing about 90% loss of the initial drug level. The process is stimulated by nitrite, which is consistent with our earlier observations using true peroxidases (1, 2). Most importantly, products of this oxidative modification of DOX are nontoxic for PC3 prostate cancer cells in vitro when compared to the parent drug, confirming that oxidation of DOX leads to its inactivation. We believe that these observations may have implications for the therapeutic efficacy of anthracyclines, as they suggest that components of sera may diminish the drug’s tumoricidal activity.

We have previously shown that DOX oxidized by LPO/H2O2/NO2− or by MPO-positive HL-60 cells/H2O2/NO2− systems is markedly less cytotoxic for human PC3 prostate cancer cells and for rat cardiac myocytes H9c2 cells in vitro (2). We were interested to find out whether oxidation of DOX by FBS also decreases its cytotoxicity. DOX was treated with the whole FBS containing NO2− (1 mM) in the presence of H2O2 (1 mM) for 1 h, after which the levels of DOX decreased by ~90%. The control sample was treated in the same fashion but with H2O2 omitted. Aliquots of the FBS containing active (sham treated) and oxidized DOX were added to PC3 cells at equivalent volumes and incubated for 10 days after which their clonogenic survival was determined. Figure 9 shows that clonogenic survival is much higher in cells treated with oxidized DOX than with the intact drug. Thus, oxidation of DOX catalyzed by serum causes inactivation of the drug.

**Figure 8.** DOX is oxidized by metHb/H2O2 in pH 7.0 buffer. (A) Absorption spectra in the main panel are from DOX (10 μM) reacting with metHb (1.3 μM) and H2O2 (0.33 mM) and NaNO2 (0.3 mM). Spectra (a) DOX alone, (b) Hb alone, (c) DOX and Hb. Spectra (d)–(n) were recorded in 1 min intervals following H2O2 addition. Inset: Time course of DOX oxidation, measured as absorption changes at 480 nm, recorded with NaNO2 omitted, NaNO2 present (0.3 mM), and Hb omitted for traces (a), (b), and (c), respectively. (B) Reaction of DOX with preformed ferrylHb. Spectrum (a) metHb (~50 μM) alone; (b) spectrum of ferrylHb recorded 3 min after addition of H2O2 (1 mM). To this ferrylHb was added DOX (10 μM) and spectra were recorded every 1 min (c)–(k). Spectrum l was recorded 3 min after (k).

**Figure 9.** Cytotoxicity of DOX for PC3 prostate cancer cells is lost upon oxidation. DOX (93 μM) was treated for 1 h with nitrite (1 mM) in full serum in the presence or absence of H2O2 (1 mM). Next, catalase (500 units/mL) was added to all samples, which were then filtered and the amount of DOX remaining was determined spectrophotometrically at 480 nm. During this time the concentration of DOX decreased to 6.7 and 83 μM, for the complete system and the system with H2O2 omitted, respectively. Aliquots of the FBS containing active (sham treated) and oxidized DOX were added to PC3 cells at equivalent volumes. The cells were exposed to DOX and its degradation products for 10 days after which their clonogenic survival was determined. Results shown are mean ± SE from three separate studies. DOX inactive and DOX active data are for cell survival determined for cells exposed to oxidized and intact DOX, respectively.
We also considered two other heme proteins as possible candidates for the serum peroxidase, namely, MPO and LPO, both of which have been shown to support oxidation of anthracyclines by \( \text{H}_2\text{O}_2 \) in the presence of nitrite \((1, 2)\). Human serum contains MPO in the range from 5.4 to \(~140\ \mu\text{g/L}\) depending on age, sex, and apparent genetic factors \((9)\). MPO levels ranging from 1.5 to \(~1100\ \mu\text{g/L}\) have been measured in human serum from patients with acute coronary syndromes and, as the authors elaborated, these levels were measured in patients that had received heparin \((10)\). Therefore, MPO levels may have been elevated due to release from endothelium into the circulation. Additionally, it has been found in in vivo studies that MPO can be localized during inflammation in vascular endothelial cells and sub-endothelial spaces where it may modulate NO availability \((23–25)\). These studies clearly demonstrate that MPO is present in human serum at measurable levels. In contrast to MPO, the presence of LPO in FBS is less likely.

FBS is used routinely in many cell culture studies examining cytotoxicity of anticancer drugs. Results of our studies show that attention has to be paid to the possibility that FBS components including Hb and MPO. It has been reported that cofactors other than \( \text{NO}_2^- \) may act catalytically in such processes as demonstrated for acetaminophen or salicylic acid in LPO and MPO systems \((3, 4)\). An earlier study on the interaction of DOX with rat serum indicated that DOX undergoes one-electron reduction to a radical that could be detected by EPR, but only in anaerobic media \((26)\). The reaction required NAD(P)H as electron donors; inhibitors of flavoenzymes inhibited radical formation. In addition, the EPR signal of the DOX radical was absent in the presence of air. Clearly, this metabolism is different from that observed in the present study. Specifically, the reductive metabolism of DOX and its redox cycling do not cause modification of the drug’s chromophore; thus, its redox properties are maintained. In contrast, oxidation of DOX leads to its degradation, which clearly is an irreversible process.

How relevant are these observations to the therapeutic action of DOX? Since during chemotherapy DOX is administered intravenously, it may interact directly with various blood components including Hb and MPO. It has been reported that a significant portion of administered drug is bound to circulating erythrocytes \((27)\). The question is whether this interaction can cause inactivation of DOX. Results of our study suggest that oxo-ferriHb, which can be generated from either metHb or oxyHb (HbO2) by the action of H2O2 \((13)\), oxidizes DOX. H2O2 is generated in vivo during normal physiological processes, but its level is markedly increased under oxidative stress conditions. OxoHb undergoes autoxidation forming metHb and superoxide, which ultimately produces H2O2 \((28)\). Nitrite oxidizes HbO2 to metHb in a reaction that also produces H2O2 \((29)\). In addition, in cellular systems the peroxide is generated by anthracyclines themselves via aerobic redox cycling \((8)\). Erythrocytes are the major reservoir of nitrite in human blood \((30)\). The natural level of nitrite in erythrocytes has been determined to be 288 \pm 47 nM, much higher than that in plasma or whole blood \((30)\). The levels of nitrite are even higher in disease states due to increased activity of nitric oxide synthase. Therefore, a complete enzymatic system necessary for inactivating DOX may exist in the blood. It has to be emphasized, however, that in vivo H2O2 is rapidly removed by catalase and/or glutathione peroxidase, which limits the availability of the peroxide for other reactions. Oxidation of DOX may also be inhibited by endogenous antioxidants such as ascorbate or glutathione. Thus, oxidative inactivation of DOX in vivo may occur under specific conditions characterized by the absence, or sufficiently low levels, of antioxidant systems. The report that 3MePA accumulates in tissues, especially in the heart, of mice administered DOX strongly supports occurrence of oxidative degradation of DOX in vivo \((5)\). In this case degradation of DOX was linked to its oxidation by myoglobin, a protein especially abundant in the heart \((5, 19)\). It needs to be emphasized that levels of antioxidant enzymes are particularly low in the heart \((31, 32)\).

Our observations may be pertinent to clinical pharmacology and toxicology of anthracyclines. Animal and human studies have shown a large disparity in the drugs’ recovery from various tissues determined by fluorescence and radioactivity measurements \((33, 34)\). The lower recovery of the fluorescent materials was interpreted as indicating either binding of drugs to cellular components in a fashion that does not allow for their extraction or, more likely, that they undergo a metabolic transformation to nonfluorescing products. DOX, its major metabolite doxorubicin, and DOX-derived aglycones show similar fluorescence excitation and emission spectra; they all possess the same intact original chromophore. However, products of the oxidative transformation of DOX, 3MePA and 3MeSA, do not absorb at 480 nm and therefore do not fluoresce when excited at this wavelength. Our observations lend strong support to the idea that the low recovery of the fluorescent metabolites may indeed be the consequence of an extensive degradation of DOX and suggests that this process may be oxidative in nature. Finally, it should be mentioned that results described in this report on DOX are fully applicable to a related anthracycline, daunorubicin (not shown). Further studies are needed to elucidate the role of the oxidative pathway in the metabolic inactivation of anthracyclines and assess biological properties of their degradation products in vivo.

**Summary**

We have shown that serum in the presence of H2O2 oxidizes DOX to nontoxic products and that nitrite markedly stimulates this reaction. The major role in this metabolism of DOX has been attributed to hemoglobin, which is present naturally in sera. Occurrence of such a reaction in vivo would substantially diminish the therapeutic efficacy of the drug. This study emphasizes that addition of H2O2 to media containing FBS will stimulate peroxidase-type of reactions, which may affect cytotoxic properties of studied compounds. Inactivation of anthracyclines described in this study is only one example of possible complications resulting from such reactions.

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**References**


