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Redox transformations of quinone antitumor drugs in liver microsomes

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The hydroxyl radical has been spin trapped in microsomal and purified NADPH-cytochrome P-450 reductase systems in the presence of adriamycin, daunomycin and mitomycin C. The presence of a lag period in quinone-stimulated spin-adduct formation is associated with oxygen removal upon its reduction to H_2O_2. The hydroxy radical generation has been stimulated by the Fe-EDTA complex and completely inhibited by catalase. The mechanism of redox transformations of anthracyclines in a microsomal system has been proposed The single electron reduced quinone-containing anticancer antibiotics play the following roles: (i) they reduce oxygen to H_2O_2 and (ii) they reduce the ferric ions necessary for H_2O_2 decomposition with hydroxyl radical formation.

Anthracycline antibiotic; Spin trapping; Hydroxyl radical; ESR

1. INTRODUCTION

Anthracycline quinones such as adriamycin, daunomycin and *N*-heterocyclic quinone mitomycin C are presently the most potent anticancer drugs in clinical use [1]. Unfortunately, application of these agents is limited by their side effects and primarily by their considerable cardiotoxicity [2]. As known, in sarcosomes, microsomes and mitochondria quinones undergo a single electron reduction to semiquinones and then are reoxidized by dioxygen to generate superoxide and hydroxyl radicals [3–5]. While some investigators believe the therapeutic and cardiotoxic effects to be caused by intercalation of the agents into DNA and by the action of semiquinones [6,7], others associate them with the action of oxygen radical forms. In particular, it has been reported that the ability of anthracyclines to split DNA and their cardiotoxicity correlate with oxygen radical generation [8,9], and that the action of highly active complexes of adriamycin-Fe and daunomycin-Fe results in hydroxyl radical formation [10]. The reaction mechanism leading to quinone-induced hydroxyl radical formation is still not clear. In recent direct experiments with flash photolysis we have shown that a widely discussed interaction between semiquinones and hydrogen peroxide which results in hydroxyl radicals [5,11,12] does not actually occur [13]. A general scheme for redox transformation of synthetic quinones in microsomal electron transfer chains has been formulated and verified in [14,15]. The aim of the present work is to investigate the redox transformation of antitumor quinones and the mechanism of hydroxyl radical production in the course of their reduction in microsomal systems. Using ESR and spin-trapping techniques we have revealed a dual role of drug semiquinones in hydroxyl radical generation: (i) reduction of oxygen to hydrogen peroxide; (ii) Fe^{3+} reduction to Fe^{2+} and initiation of the Fenton process.

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2. MATERIALS AND METHODS

The microsomal fraction was obtained from livers of male Wistar rats as previously described [16]. The concentration of microsomal protein and cytochrome P-450 was determined using the methods described in [17,18]. NADPH-cytochrome P-450 reductase (FP) isolated and purified as described in [19]

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was kindly supplied by Professor V.V. Lyakhovich. Its activity was determined from the rate of reduction of cytochrome *c* [20]. The microsomal reduction of antibiotics under study was followed spectrophotometrically in an anaerobic system (3 units/ml glucose oxidase, 3000 units/ml catalase and 0.1 M glucose). The reaction was carried out in a sealed cell in argon. The rate and depth of anaerobiosis were controlled polarographically. NADPH oxidation was measured from the decrease in absorbance at 340 nm [21]. Hydroxyl radical formation was determined from the ESR spectra of spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) in 5% dimethylsulfoxide (DMSO) in an ER-200D-SRC (Bruker) spectrometer. A flat sealed cell was used ($V = 200\ \mu l$).

Adriamycin and mitomycin C were obtained from Serva (FRG) and daunomycin (rubomycin) was provided by Minmedprom (USSR). The DMPO spin trap (Sigma, USA) was purified as described in [22].

3. RESULTS

Under anaerobic conditions the purified FP can reduce the quinone antitumor drugs to semiquinones, absorbance disappearing at its maximum (for adriamycin and daunomycin λ_{max} = 495 nm). It is noteworthy that during the reduction of anthracyclines, a precipitate is formed in the reaction mixture which seems to be due to aglycon accumulation [5]. Formation of spectrophotometrically detectable hydroquinone was not observed in the presence of oxygen. This is attributed to fast reoxidation of the reduced quinone forms (semi- and hydroquinones) by atmospheric oxygen [23].

In a system containing a quinone antibiotic, NADPH and FP, we measured the aerobic oxidation of NADPH as a function of quinone concentration. This oxidation was satisfactorily described by a Michaelis dependence (K_m values being 2.2×10^{-4} M, 2.5×10^{-4} M and 5.5×10^{-4} M for adriamycin, daunomycin and mitomycin C, respectively). Reasonably, the following reactions involving the quinone (Q) may be assumed to occur in the given system:

$$Q + e \longrightarrow Q^{\cdot -} \quad (1)$$

$$Q^{\cdot -} + O_2 \longrightarrow Q + O_2^{\cdot -} \quad (2)$$

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{2H^+} H_2O_2 + O_2 \quad (3)$$

It is known that the NADPH- or sodium dithionite-reduced FP is reoxidized rather slowly by molecular oxygen [24]. The antibiotic accepts electrons from FP (reaction 1) to stimulate the NADPH oxidation and reduction of oxygen to H_2O_2 (reactions 2 and 3).

As reported in [3–5] for enzymatic reduction under aerobic conditions, the quinone antibiotics generate active oxygen forms (H_2O_2, $O_2^{\cdot -}$, $OH^{\cdot}$). The hydroxyl radical in such systems results from the decomposition of hydrogen peroxide formed via reaction 3. To detect the formation of hydroxyl radicals, we used a DMPO spin-trap in the presence of 5% DMSO [25]. Interaction of a primary hydroxyl radical with DMSO yields a secondary CH_3 radical which forms a spin adduct with DMPO [26] producing an ESR spectrum of sextet structure having hyperfine splitting constants a_N = 15.6 G and $a_H\beta$ = 22.6 G [25]. The spectrum and kinetics of $CH_3^{\cdot}$ adduct formation in our system are shown in fig.1 using mitomycin C as an example. The presence of a lag period is characteristic of kinetics which may be associated with oxygen removal upon its reduction to H_2O_2 (reactions 2 and 3). This is confirmed by the following results: the lag period became shorter with decreasing initial concentration of diluted O_2

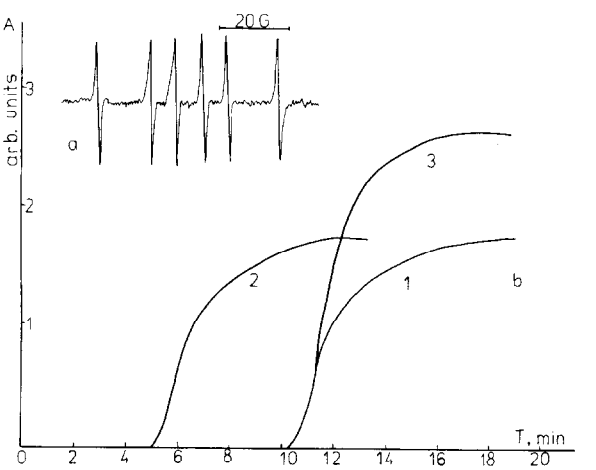

Fig.1. (a) The ESR spectrum of the $CH_3^{\cdot}$-DMPO spin adduct formed in the system containing 10^{-3} M NADPH, 0.3 IU/ml FP, 5×10^{-4} M mitomycin C, 0.1 M DMPO, 5% DMSO in 0.1 M potassium phosphate buffer (pH 7.6). The ESR settings are: field, 3474 G; sweep width, 100 G; microwave power, 19.8 mV; modulation amplitude, 1 G; receiver gain, 5×10^5. (b) The $CH_3^{\cdot}$-DMPO spin adduct accumulation kinetics measured by the intensity of sextet low-field component (a). Reaction mixture and experimental conditions are described in the cations to (a). 1, without admixture; 2, a priori solution was aspirated off by the air-argon mixture (1 : 1); 3, in the presence of 2.5×10^{-7} M Fe^{3+}-EDTA.

when the system was bubbled with an argon-air mixture (1 : 1) (fig.1). In the presence of catalase no ESR signal was detected providing evidence for hydroxyl radical generation from H_2O_2. Superoxide dismutase did not practically affect the kinetics of spin-adduct accumulation. In the absence of quinones, the spin-adduct formation was not observed either.

Of paramount importance is the question: what particular reaction is responsible for hydroxyl radical formation in our system? Many authors assume [5,11,12] that in the presence of antitumor quinones the hydroxyl radical is formed via the reaction:

$$Q^{\cdot -} + H_2O_2 \longrightarrow Q + OH^- + OH^{\cdot} \quad (4)$$

However, flash photolysis has shown that such a reaction does not take place [13].

In our opinion, the reduction of ferric ions present in the medium by semiquinone is of the greatest importance for hydroxyl radical generation:

$$Q^{\cdot -} + Fe^{3+} \longrightarrow Q + Fe^{2+} \quad (5)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad (6)$$

Reaction 5 of adriamycin and mitomycin C semiquinones with Fe^{3+} (EDTA) proceeds at high rates [23].

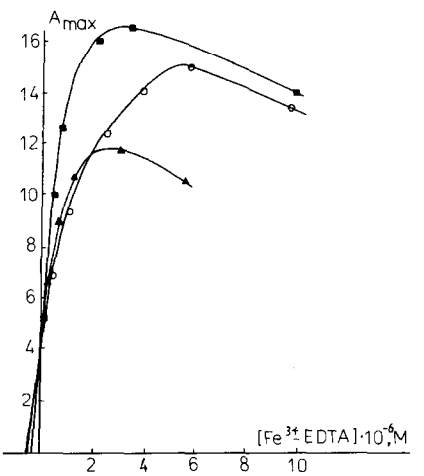

Fig.2. Dependence of maximum amplitude (A_{max}) of the ESR signal of the $CH_3^{\cdot}$-DMPO spin adduct (see fig.1a) on the concentration of the Fe^{3+}-EDTA complex. (▲—▲) Daunomycin; (■—■) adriamycin; (○—○) mitomycin C.

To verify experimentally this route, we studied the effect of the Fe^{3+}-EDTA complex on hydroxyl radical formation in our systems (fig.1). The dependence of the maximum amplitude of the ESR signal of the spin adduct (A_{max}) on the Fe^{3+}-EDTA concentrations is presented in fig.2. As seen from the figure, the approximation of these curves to zero values of A_{max} allows the estimation of the initial quantity of transition metal ions in the system corresponding to $3–5 \times 10^{-7}$ M Fe^{3+}-EDTA.

All the above characteristics for hydroxyl radical formation assisted by quinone antibiotics were also valid for an intact microsomal system. Fig.3a demonstrates the kinetics of hydroxyl radical formation in microsomes for adriamycin. A singlet with $g = 2.0036$ which rapidly decreases with time is observed after the disappearance of the ESR signal belonging to the $CH_3^{\cdot}$ adduct (fig.3b). This

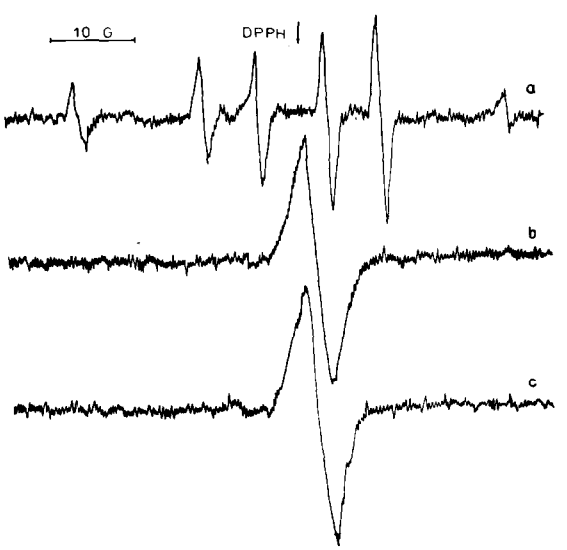

Fig.3. (a) The ESR spectrum of the $CH_3^{\cdot}$-DMPO spin adduct formed in the system containing 10^{-3} M NADPH, 0.68 mg of microsomal protein, 5×10^{-4} M adriamycin, 0.1 M DMPO, 5% DMSO in 0.1 M potassium phosphate buffer (pH 7.6). The ESR settings are analogous to those to fig.1a. Difference in the amplitude of sextet components reflects appearance and decay of spin adduct. Beginning of spectrum registration was 1.5 min after the reaction was initiated. Duration of registration of the whole spectrum was 8.3 min. (b) The ESR spectrum of adriamycin semiquinone obtained in the same reaction (a). Beginning of spectrum registration was 10 min after the reaction was started. (c) The ESR spectrum of adriamycin semiquinone obtained upon drug reduction with sodium borohydride ($NaBH_4$).

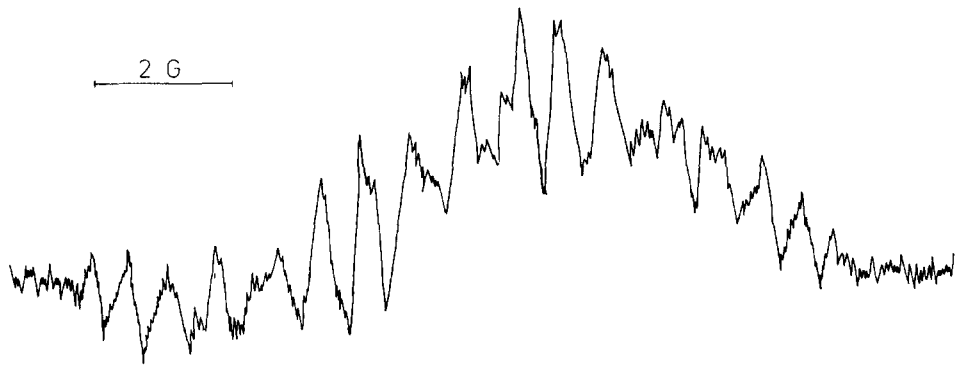

Fig.4. A hyperfine structure of an ESR spectrum of adriamycin semiquinone (see fig.3a). The ESR settings were: field, 3474 G; sweep width, 20 G; microwave power, 19.8 V; modulation amplitude, 0.1 G; receiver gain, 2.5×10^5.

new signal is identical to the ESR spectrum of adriamycin semiquinone produced by drug reduction with $NaBH_4$ (fig.3c). A hyperfine structure of this signal is shown in fig.4.

Thus in the enzymatic reduction of antitumor antibiotics in microsomes the following sequence of reactions may be proposed: first semiquinones reduce O_2 to H_2O_2 (lag period: hydroxyl radical signal is not detected) and then they reduce Fe^{3+} to Fe^{2+} which decomposes H_2O_2 to the hydroxyl radical (finally the CH_3-DMPO adduct spectrum is registered); finally a stationary concentration of semiquinones becomes large enough to detect the semiquinone ESR spectrum. Substituting NADPH by NADH in a microsomal system we observed first the CH_3-DMPO sextet and then a semiquinone singlet. However, the rate of their formation was much slower (not shown). Probably, the NADH-dependent chain of electron transfer is less effective than the NADPH-dependent one relative to the stimulation of the hydroxyl radical generation by quinone antibiotics in microsomes.

Based on the literature and our data we may assume that the ability of quinone antibiotics to generate hydroxyl radicals is realized in that part of the organism in which they may be effectively reduced, that is (i) in a microsomal system in the NADH- and NADPH-dependent chains and (ii) in a mitochondrial NADH-dependent chain [27]. Since cardiac tissue is depleted of natural antioxidants such as superoxide dismutase and catalase [18], the above assumption makes the selective cardiotoxicity of these drugs more clear.

Extraordinary antitumor potentialities of quinone antibiotics are likely to be related to the fact that in cancer cells the oxygen concentration is decreased [5,29], and under the conditions of hypoxia the semiquinones of antitumor drugs and hydroxyl radicals are formed faster and their lifetimes are longer. Assuming that the cardiotoxicity and therapeutic action of these antibiotics are of the same nature (via the hydroxyl radical) the synthesis of analogues of these drugs with increased antitumor and decreased cardiotoxic properties seems to be hardly probable.

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