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NADPH-CYTOCHROME-P450 REDUCTASE PROMOTES HYDROXYL RADICAL PRODUCTION BY THE IRON COMPLEX OF ADR-925, THE HYDROLYSIS PRODUCT OF ICRF-187 (DEXRAZOXANE)

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ICRF-187 (dexrazoxane) is currently in clinical trials as a cardioprotective agent for the prevention of doxorubicin-induced cardiotoxicity. ICRF-187 likely acts through its strongly metal ion-binding rings-opened hydrolysis product ADR-925 by removing iron from its complex with doxorubicin or by chelating free iron. The ability of NADPH-cytochrome-P450 reductase to promote hydroxyl radical formation by iron complexes of ADR-925 and EDTA was compared by EPR spin trapping. The iron-EDTA complex produced hydroxyl radicals at six times the rate that the iron-ADR-925 complex did. The aerobic oxidation of ferrous complexes of ADR-925, its tetraacid analog, EDTA and DTPA was followed spectrophotometrically. The iron(II)-ADR-925 complex was aerobically oxidized 700 times slower than was the EDTA complex. It is concluded that even though ADR-925 does not completely eliminate iron-based hydroxyl radical production, it likely protects by preventing site-specific hydroxyl radical damage by the iron-doxorubicin complex.

KEY WORDS: ADR-925, ICRF-187, EPR, hydroxyl radical, iron, cytochrome-P450 reductase.

INTRODUCTION

ICRF-187 (dexrazoxane) has been shown in clinical trials[1] to be highly effective in preventing what is likely an oxygen radical-derived iron-dependent doxorubicin-induced cardiotoxicity.[2,3] ICRF-187 is the (+)-(*S*)-enantiomer of ICRF-159 (razoxane), which upon full hydrolysis[4] (Figure 1) yields ADR-925, the (*S*)-optical isomer of ICRF-198. ICRF-198, which has a structure similar to EDTA, is also a powerful metal ion chelating agent.[5] The uncharged ICRF-159 has been shown to cross cell membranes.[6] Thus, ICRF-187 likely acts by diffusing into the cell, hydrolyzing to its active rings-opened metal ion binding form, and either chelating free iron or displacing iron from the iron-doxorubicin complex.[7] It is well known that reaction of H_2O_2 with the Fe^{2+}-EDTA complex is able to produce hydroxyl radicals or a hydroxyl radical-like product.[8] Thus, the question arises as to whether the iron-ADR-925 complex can also produce hydroxyl radicals and if so at what rate, and why they are not as damaging as radicals produced by the iron-doxorubicin complex.

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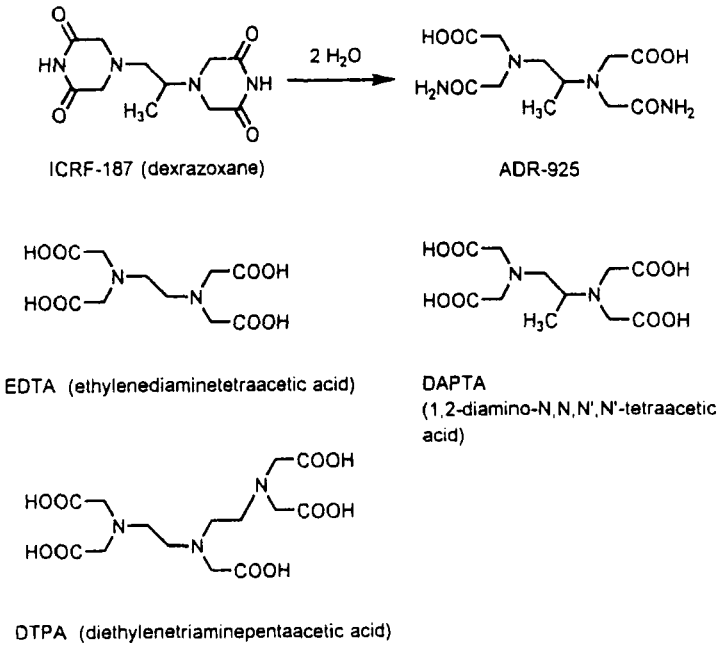

FIGURE 1 ICRF-187 and its rings-opened hydrolysis product ADR-925 and other chelating agents used in this study.

In an unrelated study[9] we demonstrated by EPR spin trapping that NADPH-cytochrome-P450 reductase promoted hydroxyl radical formation by the iron(III) complex of the mycotoxin kidney carcinogen ochratoxin A.

MATERIALS AND METHODS

The flavoprotein NADPH-cytochrome-P450 reductase was prepared and purified as described before[9] from purified rat liver microsomes and yielded a specific activity of 18,000 units per mg of protein. The spin trap DMPO (5,5-dimethyl-1-pyrroline N-oxide, Sigma, St. Louis, MO) was purified over charcoal and assayed as described.[10] The other reagents were as follows: catalase (bovine liver, Sigma), DTPA (diethylenetriaminepentaacetic acid, Sigma), NADPH (Sigma), Tris (Ultrapure, BDH Chemicals Ltd., Poole, England), $Fe(NH_4)_2(SO_4)_2$ (Aldrich, Milwaukee, WI), $Na_2EDTA \cdot 2H_2O$ (BDH), DAPTA (1,2-diaminopropane-N,N,N′, N′-tetraacetic acid, Aldrich), $FeCl_3 \cdot 6H_2O$ (Baker, Phillipsburg, NJ).

The EPR spectra were obtained in 20 mM Tris buffer (pH 7.6) and were recorded at room temperature in identical quartz capillary tubes on an X-band Bruker ESP-300 spectrometer, using 100 kHz modulation frequency. The spectra shown are the arithmetic sum of 5 consecutive spectra collected over a total time of 3.5 min. The relative radical concentrations were determined by double integration of the left-most peak of baseline-corrected spectra. The Fe^{3+} complexes of ADR-925 and EDTA for

the EPR studies were prepared under acidic conditions (1.5 mM HCl) to prevent precipitation of insoluble hydroxides, and were then diluted into the Tris buffer. A 20% molar excess of the chelating agent over Fe^{3+} was used to ensure that only complexed iron was present. The oxidation of the Fe^{2+} complexes was followed spectrophotometrically in Tris/KCl buffer (50 mM Tris/150 mM KCl, pH 7.4) on a computer-controlled Cary 1 double beam spectrophotometer thermostatted at 25°C. Absorbances at various fixed wavelengths were extracted from families of spectra for non-linear least squares curve fitting. The Fe^{2+} complexes were prepared by the direct addition of small amounts of $Fe(NH_4)_2(SO_4)_2$ to the chelating agent in air-saturated buffer in the spectrophotometer cell.

RESULTS

EPR Spin Trapping in the NADPH-Cytochrome-P450 Reductase System

The EPR spectra (Figure 2), obtained in the presence of 5% ethanol, demonstrated the characteristic 1:1:1:1:1:1 6-line spectrum of the carbon-centered DMPO-CH(OH)-CH_3 adduct.[11] The secondary spin trap method[11] was used as it allows the hydroxyl radical produced to be distinguished from DMPO-OH formed from the decay of DMPO-$O_2^{\cdot-}$ produced from the reaction of superoxide with DMPO. The

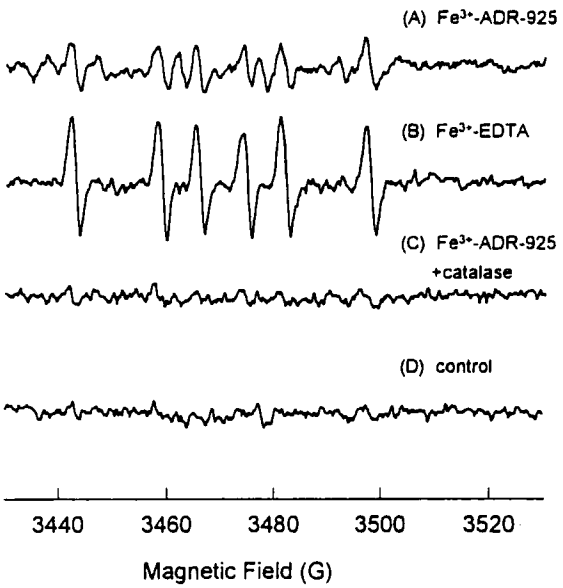

FIGURE 2 (A): The EPR spectrum obtained after the Fe^{3+}-ADR-925 complex (20 μM Fe^{3+}, 24 μM ADR-925) in Tris buffer (pH 7.6) was incubated for 11 min with NADPH-cytochrome-P450 reductase (2.4 μg protein/ml), NADPH (1.0 mM), DMPO (100 mM), and ethanol (5% v/v). (B): as in (A) above but with Fe^{3+}-EDTA instead of Fe^{3+}-ADR-925. (C): As in (A) with Fe^{3+}-ADR-925, but with the addition of catalase (0.1 mg/ml). (D): control experiment, all components as in (A) above, but in the absence of any added Fe^{3+}-ADR-925. The instrumental settings were as follows: microwave frequency 9.8 GHz, microwave power 6.3 mW and modulation amplitude 2 G.

hyperfine splitting constants produced by Fe^{3+}-ADR-925 (Figure 2A) were measured to be $A_N = 16.0$ G and $A_H = 23.0$ G and compare well with values previously reported.[11] The EPR spectrum (Figure 2A) produced by the Fe^{3+}-ADR-925 complex also shows the presence of a small amount of 1:2:2:1 4-line spectra of what is either superoxide-or hydroxyl radical-derived DMPO-OH. When catalase (0.1 mg/ml) was included in the Fe^{3+}-ADR-925 reaction mixture (Figure 2C), the hydroxyl radical-derived 6-line spectrum was almost completely abolished, indicating that there is a H_2O_2-dependent path in this system. The double integrations yielded, based on a relative radical concentration of one for Fe^{3+}-ADR-925 (Figure 2A), a value of 0.2 for the catalase-containing Fe^{3+}-ADR-925 system (Figure 2C). By comparison, the Fe^{3+}-EDTA system (Figure 2B) and the control (Figure 2D) had values of 6.2 and 0.7 respectively.

Spectrophotometric Measurements of the Aerobic Oxidation of Fe^{2+} -chelates

Since the mechanism for hydroxyl radical production in the system used in this study likely involves the reduction of H_2O_2 by the Fe^{2+} complex, it was decided to compare the aerobic oxidation of several related Fe^{2+} complexes to see if their rates of aerobic oxidation could explain the differing rates of hydroxyl radical production shown in Figures 2A and 2B. All of the Fe^{2+} complexes studied (Table 1) displayed an increase in absorbance at 300 nm, corresponding to the formation of the Fe^{3+} complex. This increase in absorbance with time was approximately exponential in nature. Non-linear least squares analysis with a 3-parameter exponential equation was used to fit the data to give the approximate k_{obs} shown in Table 1.

TABLE 1
Rates of aerobic oxidation of Fe^{2+}-complexes determined spectrophotmetrically at 300 nm in Tris/KCl buffer (pH 7.4)

Fe^{2+}-complex	k^a_{obs} (min^{-1})	Relative rate
Fe^{2+}-ADR-925[b]	0.010	1
Fe^{2+}-EDTA	7.2	720
Fe^{2+}-DTPA	0.069	6.9
Fe^{2+}-DAPTA	7.3	730
Fe^{2+}	0.041	4.1

[a] Average of 2 determinations that agreed to within 10% of each other. The complex concentration (1:1) was 100 μM.
[b] In oxygen-saturated solution this value increased to 0.025 min^{-1}.

DISCUSSION

In a mechanism similar to that proposed for hydroxyl radical production by the Fe^{3+}-ochratoxin A complex,[9] which was based on the mechanism put forth by Lai *et al.*,[12] the hydroxyl radical may be produced by a mechanism that may involve some or all of the following reactions:

$$E_{ox} + NADPH \rightarrow E_{red} + NADP^+ \quad (1)$$

$$E_{red} + O_2 \rightarrow E_{ox} + O_2^{\cdot -} \quad (2)$$

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

$$O_2^{\cdot -} + Fe^{3+}\text{-}L \rightarrow Fe^{2+}\text{-}L + O_2 \quad (4)$$

$$E_{red} + Fe^{3+}\text{-}L \rightarrow E_{ox} + Fe^{2+}\text{-}L \quad (5)$$

$$Fe^{2+}\text{-}L + H_2O_2 \rightarrow Fe^{3+}\text{-}L + HO^{\cdot} + OH^- \quad (6)$$

where E_{ox} and E_{red} are the oxidized and reduced forms of NADPH-cytochrome-P450 reductase, respectively, and L is the chelating ligand. The results obtained in the presence of catalase (Figure 2C) are consistent with the scheme above. The scheme above may be considerably simplified, as there are a variety of other reactions that the iron complexes may take part in.[13]

The data of Table 1 show that the Fe^{2+} complex of the diacid-diamide ADR-925 is aerobically oxidized much more slowly (720 times slower than for the EDTA complex) than any of the other tetraacid or pentaacid chelating agents studied. However, Fe^{2+}-DAPTA, the tetraacid derivative of ADR-925, is oxidized at about the same rate as Fe^{2+}-EDTA, which is also a tetraacid. But hydroxyl radical production measured by spin trapping is only 6.2 times slower than for the EDTA complex. This indicates that if there is any superoxide production through reaction of oxygen with Fe^{2+}-ADR-925, this reaction is not significant in the production of hydroxyl radicals in this system. Thus, in this system, oxidation of E_{red} in reaction (2) is likely the main source of superoxide.

It is known that for iron complexes to produce hydroxyl radicals there is a stringent requirement for a free-iron coordination site.[14] The 7-coordinate Fe^{3+}-EDTA has a coordinated water,[8,14,15] but Fe^{3+}-DTPA does not.[14] Interestingly, the Cu^{2+}-EDTA complex also has a free coordination site,[16] but Cu^{2+}-ICRF-198 does not.[17] The oxidation rates for Fe^{2+}-EDTA and Fe^{2+}-DTPA are similar to those previously measured.[14]

Winterbourn[18], using both DMSO oxidation and deoxyribose oxidation, has shown that the iron complex of ADR-925 in a variety of superoxide-producing systems can produce hydroxyl radicals. In a non-cycling system containing H_2O_2, the Fe^{2+}-ADR-925 complex was found to produce more hydroxyl radical (1.5 ×) than the Fe^{2+}-EDTA complex, though this factor was significantly reduced (to 0.9 ×) when the iron complexes were present only in catalytic amounts in a reducing system.[18] It was only with the superoxide-driven reaction that the ADR-925 complex gave a lower hydroxyl radical yield (0.26 and 0.71 ×) than for the EDTA complex. These latter results are more in accord with the results of this study in which the ADR-925 complex produced hydroxyl radical at a significantly lower rate (0.16×) than did the EDTA complex.

It has been shown that ICRF-198 substantially inhibits iron-induced lipid peroxidation[19] and reduces the iron-doxorubicin promoted inactivation of NADH-cytochrome *c* reductase and cytochrome *c* oxidase on submitochondrial particles.[7] The iron-doxorubicin complex likely inactivates the respiratory enzymes through hydroxyl radical-initiated lipid peroxidation caused by self-reductive activation of the iron-doxorubicin complex,[3] that occurs while the iron-doxorubicin complex is strongly bound to the submitochondrial particles.[20] The fact that strong chelating agents such as ADR-925 or EDTA prevent this inactivation is because the soluble iron complexes formed from the removal of iron from its doxorubicin complex are unable to initiate site-specific hydroxyl radical damage that would otherwise occur when the iron-doxorubicin complex is bound to the submitochondrial particle. The hydroxyl radical, being an extremely reactive species, is unlikely to cause much damage to

cellular components unless it is very close to or actually bound to that component. Thus ICRF-187 may be able to prevent iron-based oxidative damage *in vivo* due to the low rate at which the Fe^{3+}-ADR-925 is reduced;[19] and, as shown in this study, the low rate of hydroxyl radical production by the iron-ADR-925 complex and the low rate at which Fe^{2+}-ADR-925 is aerobically oxidized; and as importantly, the ability of ADR-925 to change the site at which hydroxyl radical is produced.

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