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SHORT COMMUNICATION

Electron Paramagnetic Resonance Spectrometry Evidence for Bioreduction of Tirapazamine to Oxidising Free Radicals Under Anaerobic Conditions

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ABSTRACT. Tirapazamine (SR 4233) is a bioreductive antitumour drug in Phase III clinical trial which is activated in hypoxic tumour regions to generate a cytotoxic species. Electron paramagnetic resonance (EPR) spectrometry was used to investigate directly the formation of free radicals as the result of tirapazamine reduction by NADPH-supplemented liver microsomes. Under anaerobic conditions, the tirapazamine nitroxide free radical EPR signal was not evident over a range of rat or human liver microsomal protein (1–5 mg) concentrations. However, in combination with 1,1′,5,5′-dimethylpyrolline-1-N-oxide (DMPO), a spin trap for short-lived free radicals, tirapazamine resulted in formation of a 1:1:1:1:1:1 spectrum with hyperfine splitting A_N = 15.8 G A_H = 22.3 G consistent with generation of DMPO-R, a carbon-centered radical adduct. Addition of DMSO increased the signal intensity of the carbon-centred radical by at least twofold. The hyperfine splitting constants associated with DMPO-R could be indicative of a tirapazamine carbon-centred radical per se or, more likely, carbon radicals from endogenous materials (or DMSO) in the biological matrix as a result of oxidative attack by the tirapazamine primary radical. Formation of DMPO-OH, the hydroxyl radical spin adduct, by tirapazamine in the absence of air indicates that liberation of a hydroxyl radical may be a consequence of tirapazamine bioreduction under anaerobic conditions. The reactivity of tirapazamine free radicals with endogenous microsomal substances to generate reactive carbon-centred radicals indicates that tirapazamine may disrupt a wide range of cellular activities. BIOCHEM PHARMACOL **60**;12:1933–1935, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. EPR; ESR; spin trap; tirapazamine; bioreduction; free radical

Tirapazamine is an anticancer agent that is considerably more toxic to cells in a hypoxic (low oxygen) environment [1] and is currently in Phase II and III clinical trials (e.g. [2]). Cytochrome P450 reductase is predominantly responsible for activation of tirapazamine and correlates with cytotoxicity. The formation of the tirapazamine reactive intermediate is generally inferred by detection of the tirapazamine mono-N-oxide (SR 4317). This can result from sequential two-electron reduction of tirapazamine via the tirapazamine free radical, a one-electron reduced intermediate (reviewed in [3]). The absolute identity of the tirapazamine reactive species generated enzymically is still not certain; early reports suggested that a protonated tirapazamine neutral free radical generates DNA free radicals via hydrogen atom abstraction which, by unspecified mechanisms, cleaved DNA [4]. There is one report using electron paramagnetic resonance spectrometry that shows, in rat liver microsomes, evidence for a tirapazamine nitroxide radical anion [5]. The present study attempts to characterise the nature of the tirapazamine free radical(s) generated anaerobically using electron paramagnetic resonance spectrometry. The results support the presence of an oxidising radical following enzymatic reduction of tirapazamine.

MATERIALS AND METHODS

Rat liver microsomes were prepared from Sprague–Dawley adult male rat livers by ultracentrifugation using standard procedures and stored at −80° until required. Incubations comprised microsomes (1–5 mg/mL), tirapazamine (4.2 mM in water), glucose 6-phosphate (type XXIII) 10 mM with glucose-6-phosphate dehydrogenase (Type XV, 13 units/mL), and NADPH (1 mM) as an NADPH-generating system. Tris–HCl buffer (pH 7.4, 0.01 M) was treated with Chelex ion-exchange resin before use to remove adventitiously present metal ions and hence minimise endogenously generated hydroxyl radicals. Tris buffer was added to make a final incubation volume of 500 μL. NADPH was added immediately prior to placing the incubate in an EPR quartz flat cell. In anaerobic experiments, all solutions were degassed with oxygen-free nitrogen prior to mixing and the final incubate was further degassed. Transfer of the incuba-

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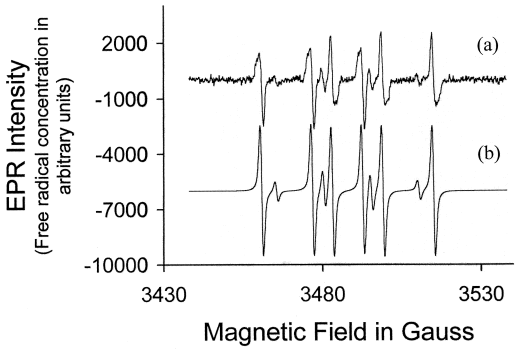

FIG. 1. (a) EPR spectrum of carbon-centred radical adducts (DMPO-R) generated by tirapazamine in NADPH-supplemented rat liver microsomes under nitrogen (b) simulation of spectrum (a). EPR spectrometer frequency 9.791 GHz; microwave power 19.92 mW; centre field set 3488 G, sweep width 100 G; modulation amplitude 0.5 G; time constant 20.48 msec; sweep time 83.8 sec; receiver gain 6.32 × 10^4.

tion mixture to the EPR flat cell (see below) was carried out under a nitrogen atmosphere. For detection of tirapazamine secondary radical products, rat liver microsomes were incubated as described above with the addition of DMPO* (100 mM) as a spin-trapping agent. In some experiments DMSO (2 μL) was added in order to investigate the nature of the secondary radical products formed.

For EPR spectrometric analysis, the incubation mixtures containing rat liver microsomes were aspirated into a quartz flat cell and immediately analysed at room temperature (approx. 22°) by a Bruker EMX X-band EPR spectrometer fitted with a T_{102} rectangular cavity. Data were collected using WIN EPR software and EPR spectra analysed using Symfonia software.

RESULTS AND DISCUSSION

Rat liver microsomes were used as a rich source of cytochrome P450 reductase in order to define the experimental conditions required to generate tirapazamine free radicals in biological samples. Figure 1 shows that tirapazamine metabolism by liver microsomes under anaerobic conditions in the presence of DMPO as a spin trap directly resulted in formation of a 1:1:1:1:1:1 spectrum with hyperfine splitting A_N = 15.8 G A_H = 22.3 G. This is consistent with generation of an adduct of a carbon-centred radical with DMPO (DMPO-R). A low-intensity 1:2:2:1 spectrum, A_N = 15.0 G, A_H = 14.9 G, was also evident. This latter spectrum may have been initially due to residual oxygen, under the stoppered EPR flat cell incubation conditions employed, giving rise to small amounts of spin-trapped hydroxyl radicals which are observed as DMPO-OH. Alternatively, under strictly anaerobic conditions, DMPO-OH may have arisen through direct release of the hydroxyl radical as a consequence of tirapazamine one-electron reduction, as was observed previously [6]. The results in Fig. 2 show that tirapazamine generated a DMPO-R and DMPO-OH adduct under aerobic conditions. However, these adducts were only observed at maximum intensity following complete loss of the superoxide anion adduct (DMPO-OOH; hyperfine splitting A_N = 14.1 G, A_{Ha} = 11.4 G, A_{Hb} = 1.3 G). Tirapazamine is known to undergo bioreductive redox cycling, resulting in superoxide anion formation [3, 5]. Under the experimental conditions used, this resulted ultimately (13 min) in an anaerobic environment due to consumption of residual oxygen in the ESR stoppered flat cell.

Unfortunately, the identity of the carbon-centred radicals observed by spin trapping in this study cannot be unequivocally ascertained. The hyperfine splitting constants associated with the 1:1:1:1:1:1 spectrum could be indicative of a tirapazamine carbon-centred radical. More likely, the spin adduct observed is due to carbon-centred radicals from endogenous materials in the biological matrix as a result of hydrogen atom abstraction by the tirapazamine primary radical, which may be an oxygen-centred (nitroxide) or carbon-centred radical. Attempts to observe the

* *Abbreviations:* DMPO, 1,1′,5,5′-dimethylpyrolline-1-N-oxide.

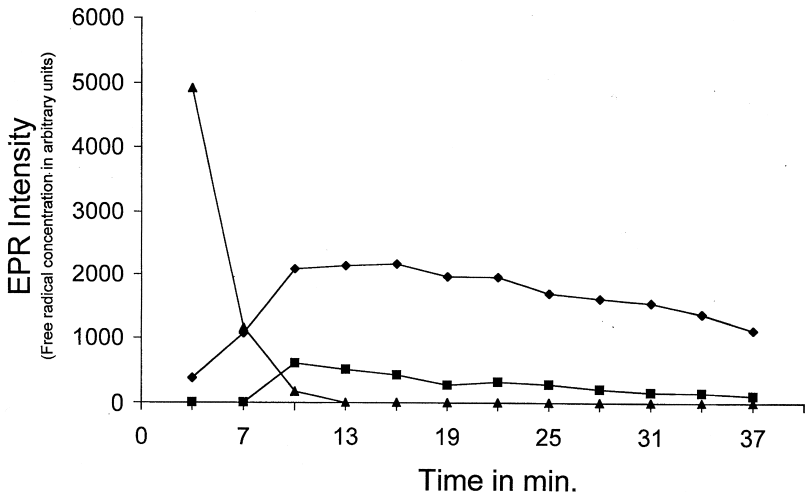

FIG. 2. Time–course for superoxide anion (DMPO-OOH) -▲-, hydroxyl radical (DMPO-OH) -■-, and carbon-centred radical (DMPO-R) -◆- formation by tirapazamine in initially aerated sealed incubates of NADPH-supplemented rat liver microsomes.

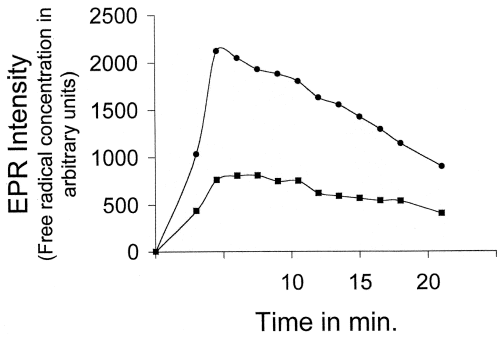

FIG. 3. Time–course of carbon-centred radical adduct (DMPO-R) generated by tirapazamine in NADPH-supplemented rat liver microsomes under nitrogen in the presence (-●-) and absence (-■-) of DMSO.

tirapazamine nitroxide free radical directly by EPR spectrometry using conditions identical to that previously reported for NADPH-supplemented rat liver microsomes [5] and at microsomal protein concentrations of 1, 2, and 5 mg per incubate were not successful. Similarly, no tirapazamine nitroxide radical was found in human liver microsomes (data not shown). However, increasing rat microsomal protein concentrations did progressively increase the tirapazamine-derived carbon-centred radical (DMPO-R adduct) (results not shown).

The increase in the tirapazamine-generated DMPO-R EPR signal intensity (Fig. 3) in the presence of DMSO supports the conclusion that bioreduction of tirapazamine under anaerobic conditions results in an oxidising free radical. Specifically, the tirapazamine free radical can be considered to oxidise DMSO, giving rise to the methyl radical that is trapped as DMPO-CH_3. The hyperfine splitting constants associated with the 1:1:1:1:1:1 spectrum of DMPO-R (see Fig. 1) are entirely consistent with this interpretation. It is of interest that the tirapazamine-derived free radical products trapped in the absence of air in this study display similar reactivity towards DMSO to that described for the protonated tirapazamine neutral radical, which decomposes by liberating a hydroxyl radical [6]. The lack of detection of the tirapazamine nitroxide radical in the present study is of concern, especially since only one report has described its detection by EPR spectrometry [5]. Another study using this technique for spin trapping tirapazamine-mediated reactive oxygen [7] does not address formation of the primary radical. Whatever the exact nature of the tirapazamine free radical, it is capable of generating carbon-centred radicals that are anticipated to undergo a variety of reactions, including DNA strand breakage, associated with cell toxicity.

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