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# ELECTRON SPIN RESONANCE STUDIES ON ISOLATED HEPATOCYTES TREATED WITH FERROUS OR FERRIC IRON

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Isolated rat hepatocytes incubated with iron salts in the presence of the spin trapping agent α-4-pyridyl-1-oxide N-tert-butyl nitrone (4-POBN) generate a clear electron spin resonance signal; this signal is not detectable in the absence of exogenous iron. The hyperfine splitting constants are identical whether ferrous or ferric iron is used. The free radical trapped does not appear to be an active oxygen species but rather a carbon-centred radical, which we here ascribe to a lipodienyl radical on the basis of its hyperfine splitting features. Support to this interpretation is lent by the fact that no such radical could be generated in hepatocytes fully protected against lipid peroxidation by pretreating the donor rats with α-tocopherol.

KEY WORDS: Iron overload, free radicals, electron spin resonance, vitamin E, lipid peroxidation.

## INTRODUCTION

In developed idiopathic hemochromatosis and in other forms of iron overload, tissue injury is preceded by the accumulation of excess iron; a major target organ is the liver.[1,2] The mechanism of tissue injury in iron overload is by no means clear, but the redox-active nature of iron is generally believed to be of fundamental importance, the ability of iron to catalyse the generation of reactive free radicals being well documented. In the healthy cell, iron is held in non-reactive, protein-bound ferric forms in order to prevent the potentially lethal consequences of iron-catalysed free radical generation. When the ability of the cell to maintain iron in this non-reactive form is exceeded, the metal may participate in, for example, catalysis of the Haber–Weiss reaction, generating the highly reactive hydroxyl radical ($OH^{\cdot}$).[3] This radical can initiate lipid peroxidation of the polyunsaturated fatty acids of cellular membranes, a chain reaction that is also accelerated by iron. Certain iron-chelates, such as ADP-iron, can initiate lipid peroxidation in liver microsomal suspensions[4] and in isolated hepatocytes,[5] and iron-overload-induced lipid peroxidation has been detected in rat liver *in vivo*.[6]

The identification of the free radical intermediates involved in iron toxicity is clearly of importance and the most appropriate method is electron spin resonance (e.s.r.) spectroscopy. When coupled to the technique of spin trapping, this method has

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proved to be capable of measuring radical production in a variety of biological systems.[7] The present study reports experiments designed to trap and identify free radical intermediates formed during the treatment of isolated hepatocytes with iron *in vitro*.

## METHODS

Male Wistar rates (Nossan, Correzana, Milano, Italy), body weight 200–250 g, were used throughout. They were fed an antioxidant-free semisynthetic diet (Piccioni, Brescia, Italy) with free access to water. Animals whose livers were to be loaded with vitamin E were injected intraperitoneally with α-tocopherol (Sigma Chemical Co., Cat. No. T3251) at 100 mg/kg, 15 h before preparation of hepatocytes. The α-tocopherol was dissolved in one volume of ethanol and diluted in 9 volumes of 16% (v/v) Tween 80 in 0.9% NaCl.[8] Hepatocytes were isolated by the collagenase perfusion method described previously;[9,10] the media used for cell isolation and incubation were as described with minor modifications to preserve the glutathione content.[11] Cell viability was assessed by the Trypan Blue exclusion test and was always in the range of 85–95%. Aliquots of hepatocyte suspension (2 ml; $1.5 \times 10^7$ cells per ml) were incubated at 37°C in stoppered 50 ml flasks in the presence or absence ADP/$FeCl_3$ (2.5 mM/0.1 mM) or $FeSO_4$ (0.1 mM) and in the presence of the spin trapping agent α-4-pyridyl-l-oxide N-tert-butyl nitrone (4-POBN; 25 mM). The incubation was stopped after 30 mins by adding 1 ml of ice-cold chloroform/methanol (2:1, v/v). After thorough mixing and a brief centrifugation, the chloroform phase was taken for e.s.r. analysis. Operating conditions were: microwave power, 10 mW;: modulation amplitude, 1 G; scan range 100 G; temperature, − 70°C. Cells were also incubated in the absence of spin trap with and without ADP/$FeCl_3$ and aliquots taken at times up to 4 h for analysis of α-tocopherol content essentially as described by Burton *et al.*[12] for liver homogenate. Hepatocytes (1 ml) were mixed with 1 ml of 50 mM sodium dodecyl sulphate and 2 ml of absolute ethanol. This was followed by rotamixing with 1 ml of n-heptane and after a brief centrifugation the heptane phase was taken for analysis by h.p.l.c. An S5-CN column (25 cm × 4.6 mm; Anachem Ltd, Luton, U.K.) was used; mobile phase was hexane:isopropanol (99:1; v/v) at 1.5 ml/min. The U.V. detector was set at 295 nm and α-tocopherol was quantitated by comparison with injected standard solutions and by use of a computing integrator.

## RESULTS AND DISCUSSION

As shown in Fig. 1, incubation of normal isolated hepatocytes with the 4-POBN spin trap in the absence of exogenous iron salts yields only a baseline e.s.r. signal. When iron salts are added, in the form of $FeSO_4$ (0.1 mM) or as ADP/$FeCl_3$ (2.5 mM/0.1 mM), well defined e.s.r. spectra are generated. Analysis of the hyperfine splitting constants (Table I) shows that the spectra are identical whether ferrous or ferric salts are used and suggests that the radical species trapped is the same in each case. The spectral features also indicate that it is not an oxygen-centred radical but rather one centred on a carbon atom. The values reported by Finkelstein *et al.*[13] for the adducts of 4-POBN with $OH^{\cdot}$ and with $O_2^{\cdot-}$ are also given in Table I for comparison. It can

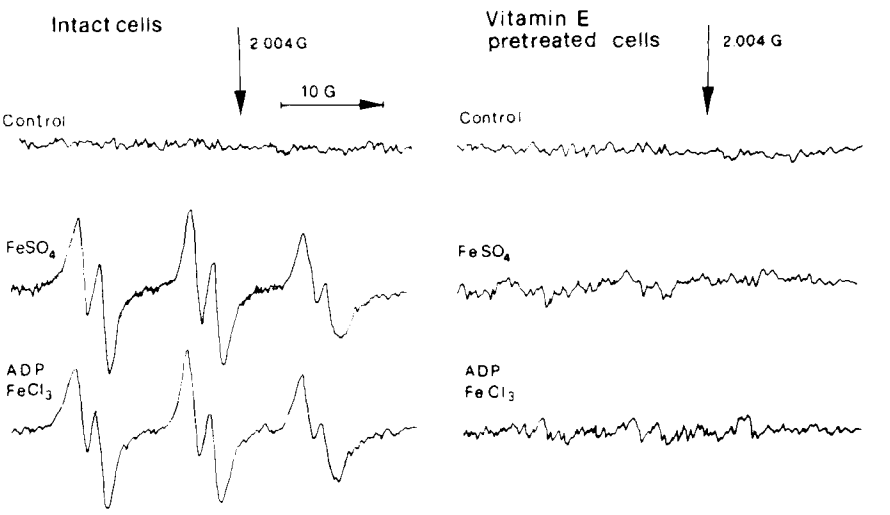


FIGURE I Electron spin resonance spectra of 4-POBN-free radical adducts extracted from whole isolated hepatocytes, from normal or vitamin E-pretreated rats, after incubation at 37°C for 30 min with and without ferrous or ferric iron.

TABLE I

Hyperfine splitting constants of the 4-POBN-free radical adducts produced in isolated hepatocytes treated with $FeSO_4$ or $ADP/FeCl_3$

| Origin of radical adduct | Hyperfine splitting contants (Gauss) | |
|---|---|---|
| *This study* | $A_N$ | $A_H$ |
| $FeSO_4$ | 14.84 | 2.87 |
| $ADP/FeCl_3$ | 14.80 | 2.90 |
| *Literature values* | | |
| (a) $L^{\cdot}$ | 15.10 | 2.94 |
| (b) $OH^{\cdot}$ | 14.97 | 1.68 |
| (c) $O_2^{\cdot-}$ | 14.16 | 1.75 |

$A_N$: nitrogen hyperfine splitting; $A_H$: hydrogen hyperfine splitting.

(a) Liver microsomes incubated with soya-bean lipoxygenase in the presence of 4-POBN.[14]

(b) Xanthine/xanthine oxidase/4-POBN; spectrum recorded in aqueous solution.[13]

(c) Photolysis of $H_2O_2$ in presence of 4-POBN; spectrum recorded in aqueous solution.[13]

be seen that, even allowing for differences in the analytical solvents, the hyperfine splitting constants are markedly different to those obtained from liver cells incubated with iron salts. In fact, the latter signals are very similar to those obtained when soya-bean lipoxygenase is incubated with rat liver microsomes in the presence of

TABLE II

Concentration of α-tocopherol in isolated hepatocytes from vitamin E treated rats: effect of incubation with ADP-$FeCl_3$

| Incubation time (h) | Control | ADP-$FeCl_3$ treated |
|---|---|---|
| | (nmols/$10^7$ cells) | |
| 0 | 85.4 (100%) | 85.4 (100%) |
| 1 | 85.6 (100%) | 85.7 (100%) |
| 2 | 80.7 (94.4%) | 81.2 (95.1%) |
| 3 | 77.3 (90.5%) | 80.1 (93.7%) |
| 4 | 73.2 (85.7%) | 72.8 (85.3%) |

*Note*: In isolated hepatocytes from untreated animals the initial α-tocopherol concentration was 9.7 nmols/$10^7$ cells.

4-POBN.[14] Accordingly, we ascribe the signal produced in the iron-poisoned hepatocytes to a 4-POBN-lipodienyl radical adduct, probably resulting from iron-mediated lipid peroxidation of unsaturated fatty acids in the cellular membranes of the hepatocytes.

This interpretation is strongly supported by the finding that pretreatment of rats such that the hepatocyte is enriched some 9-fold in α-tocopherol suppresses production of this radical to below the limit of detection (Fig. 1). Iron-induced lipid peroxidation, measured as malonaldehyde production, is also strongly inhibited by this pretreatment.[15]

Analysis of the actual α-tocopherol content of these hepatocytes shows that not only do they contain approximately 9-fold more α-tocopherol than hepatocytes from untreated animals but that this level is maintained for a relatively long period of time even when the cell is strongly stressed (Table II). Thus, even in the presence of the potent pro-oxidant ADP/$FeCl_3$ the cellular concentration of α-tocopherol is maintained at or near the initial level for incubation times well in excess of those used for spin-trapping experiments. This is probably achieved by regeneration of α-tocopherol from the α-tocopheroxy radical by direct reaction with ascorbate[16] and by repair of the vitamin radical by GSH, the latter possibly being enzyme-mediated.[17] If such repair systems are efficient, and that will depend on the relative rates of oxidizing radical production and of repair, then lipid peroxidative damage to cell membranes may be suppressed until the depletion of co-factors for α-tocopherol repair followed by depletion of α-tocopherol itself.

The experiments reported here are analogous to earlier experiments by Piette and co-workers,[18,19] who attempted to spin-trap oxygen radicals ($OH^{\cdot}$, $O_2^{\cdot-}$) in liver microsomes incubated with NADPH and iron-chelates. In those papers, evidence was provided suggesting that $OH^{\cdot}$ was produced by a reaction requiring NADPH and iron and possibly proceeding via production of $O_2^{\cdot-}$ (i.e. the iron-catalysed Haber–Weiss reaction).

In the present study, using whole liver cells, neither $OH^{\cdot}$ nor $O_2^{\cdot-}$ was trapped and there are several possible reasons for this. Firstly, these radicals may not actually be produced; some workers believe that the molecular species initiating lipid peroxidation in such systems is in fact a perferryl form of iron-complex.[20] Secondly, the putative oxygen radicals may react preferentially with cell components: $OH^{\cdot}$ will react almost indiscriminately with lipids and proteins at near diffusion-controlled rates.

Finally, the 4-POBN-OH· radical adduct is unstable and may not survive the incubation and extraction procedure.[7,21] A similar problem may explain why lipid peroxyl radicals are not detected in these experiments: the spin-trap-lipid peroxyl radical adducts are rather short lived and are very difficult to detect in biological systems (M.J. Davies, personal communication). An important aspect of the spin trapping technique to bear in mind is that the radical species most easily detectable may not be that which is most important in either quantitative or toxicological terms but merely that which is most stable. In the present study the radical trapped appears to be a carbon-centred lipid radical, probably an intermediate of lipid peroxidation rather than the sought-for primary (initiating) radical. It remains to be seen if the spin trapping technique can be suitably refined to detect oxygen radicals and/or lipid peroxyl radicals in iron-stressed hepatocytes.



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