Free Rad. Res., Vol. 27, pp. 645–656
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Free Radical Production After Exposure of Astrocytes and Astrocytic C6 Glioma Cells to Ethanol. Preliminary Results

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Accepted by Prof. S. Orrenius

(Received 11 September 1996; In revised form 15 June 1997)

Formation of the α-hydroxyethyl radical (CH3°CHOH) has already been extensively demonstrated after ethanol metabolism in the liver. Despite favourable conditions, this formation in the brain has remained speculative since there is no direct experimental evidence in intact brain cells. In this preliminary study, the formation of such a radical was observed after exposure of astrocytes and astrocytic C6 glioma cells to ethanol. These cells were studied because astrocyte integrity is essential for normal growth and functioning of neurons. The free radicals were detected by EPR spectroscopy using the spin trapping technique. Astrocytes appeared to be more sensitive than the C6 cells to free radical formation as the intensity of the signal was higher after exposure of the astrocytes and increased with time, a fact not observed after exposure of the C6 cells.

Keywords: Ethanol metabolism, free radical formation, EPR, spin-trapping, astrocytes, C6 glioma cells

Abbreviations: EPR: electronic paramagnetic resonance, DMPO: 5,5-dimethyl-1-pyrroline N-oxide, POBN: α-(4-pyridyl 1-oxide)-N-tert-butylnitrone, PBN: phenylbutylnitrone, Fe(II): ferrous iron provided by $FeSO_4$, DETAPAC: diethylene triamine pentaacetic acid, EtOH: ethanol, PBS: phospate buffer.

INTRODUCTION

Free radical generation occurs during the metabolism of various xenobiotics.[1] The potential damaging effects resulting from the production of reactive oxygen species (ROS) are prevented by a large range of anti-oxidant molecules (enzymes, vitamines, metal chelators. . . .) in normal conditions. However, in some cases (acute intoxication, for example), the balance between pro and anti-oxidant species is modified and unscavenged radicals may damage the cell.

The oxidation of ethanol via a radicalar pathway is well described. Ethanol reacts with hydroxyl free radicals, thus generating the α-hydroxyethyl radicals (CH3°CHOH) whose

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production was demonstrated *in vitro* in a number of chemical[2] and biological systems.[3,4,5] Moreover, different authors showed *in vivo* α-hydroxyethyl radical formation after ethanol intake. Most of these experimental studies have been conducted on the liver, as this organ is the main target for xenobiotic metabolism.[6,7,8,9] However, ethanol intake induces behavioural changes and alterations of the central nervous system, indicating that brain is also an important target. Among the mechanisms responsible for ethanol neurotoxicity which remain largely unknown, the role of free radicals is discussed.[9] Indeed, the conditions for free radical production are present in the brain despite a poorer enzymatic equipment than in the liver. For example, cytochrome P-450 which is responsible for the production of ethanol-derived free radicals, has been identified in the brain as well as the cytochrome P-450IIE1, its isoform more specifically implicated in ethanol metabolism.[10,11,12] ROS formation has been described in the brain after ethanol intake[13,14] concomitantly with an increased cytochrome P-450 concentration.[11,12] Finally, α-hydroxyethyl radical formation has been demonstrated in brain microsomes exposed to ethanol, by Electronic Paramagnetic Resonance (EPR) spectroscopy using the spin-trapping technique.[5] However, no direct evidence is available from studies conducted in intact brain cells.

The present study therefore aimed at demonstrating the ability of astrocytes and astrocytic C6 glioma cells in culture to produce free radicals derived from ethanol metabolism. These cells were studied because astrocyte integrity is essential for normal growth and functioning of neurons.[15] The deleterious effects of the free radicals produced by these cells could thus affect the functioning of whole brain. C6 glioma cell lines were chosen because of their similarities with astrocytes and their long life. Furthermore, the use of cell cultures avoids the interference of certain secondary effects either due to undernutrition frequently observed after animal exposure to prolonged and heavy ethanol absorption, or to indirect cerebral consequences of liver ethanol metabolism. Finally, the free radicals were detected by EPR spectroscopy using the spin trapping technique.

MATERIALS AND METHODS

Cell Cultures

Primary astroglial cells

Pregnant Sprague-Dawley rats were obtained from IFFA-CREDO (L'Arbresle, France). Primary cultures of astrocytes were prepared aseptically from cerebral hemispheres of 1- and 2-day-old pup rats according to previously described methods[16,17] with a few modifications. After removing adherent meninges, the brains were minced with scapels and incubated at 37°C for 30 min in phosphate-buffered saline (PBS) containing 0.18% D-glucose (Sigma), 0.4% trypsin (Gibco), 0.1% DNAse IV (Sigma).

After two washes in PBS, the action of trypsin was stopped by addition of a trypsin inhibitor (0.2%, Sigma). Cells were then mechanically dissociated by gentle trituration with a fire-polished, siliconized Pasteur pipette. The suspension was centrifuged (8 min, 160 g) twice in PBS and the pellet re-suspended in 50% V/V HAM's F-12 (Gibco)/Minimal Essential Medium (MEM, Gibco) with 10% fetal calf serum (FCS, Boehringer Mannheim), 0.03% L-glutamine (Gibco), 0.6% D-glucose (Sigma), 0.11% sodium bicarbonate (Sigma), 0.12% HEPES buffer (Sigma), 0.1 mg/ml-100U/ml penicillin-streptomycin (Pen/Strep) (Boehringer Mannheim), and 2.5 mg/l amphotericin B (Boehringer Mannheim).

Aggregates were removed by filtration through a 100-μm followed by a 20-μm nylon mesh. Cell number and viability were assessed using a blue trypan exclusion test.

Tissue culture flasks (Costar, 25 cm2, Dutscher) were filled with 2.5 ml of 0.001% polylysine (Sigma) for 30 min, emptied and air dried.

Dissociated cells were plated at a density of 4×10^4 viable cells per cm^2 in the usual medium

and cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed 2 h after seeding and then every 3 days. The cultures reached confluency after 7 days *in vitro* (DIV).

Fourteen days after seeding, the monolayers were composed of at least 95% astrocytes, as demonstrated by positive immunostaining with antiserum to α-GFAP, an astrocyte marker.

Astrocytic glioma cell lines

Glial cell lines (C6 glioma cells) were initially provided by the laboratory of Pr Feuerstein (Département de Neurophysiologie, Faculté de Médecine, Grenoble), at passage 52. Cells were used between passages 54–62. They were initially plated in a 25 cm^2 flask at a density of 2×10^5 cells/cm^2 in a culture medium consisting of 80% Dulbecco's modified Eagle's medium (D-MEM, Gibco), 20% FCS, and 100 U/ml Pen/Strep. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Every 3 days, cells were harvested with 0.25% trypsine, resuspended in culture medium (90% D-MEM, 10% FCS, 100 U/ml Pen/Strep), and seeded at a density of 2×10^4 cells by cm^2.

Cell Viability and Protein Determination

Viability of astrocytes and C6 glioma was determined by the trypan blue (0.1%) exclusion test. Protein determination was achieved according to the method of Lowry adapted to protein quantification in cell culture,[18] using bovine serum albumin as standard.

Conditions of Ethanol Exposure

To avoid the non-detection of the free radicals due to the bioreduction of spin-adducts by reducing species present in the culture medium such as ascorbate, thiol functions, enzymes which can transform by a one-electron reduction the spin adduct into an EPR-invisible hydroxylamine,[19,20] the ethanol exposures were performed in phosphate buffer (PBS) pH 7.4.

Flasks were rinced twice with PBS. The cells were then incubated for various times (15 to 120 min) at 35°C in PBS containing 50mM POBN (Aldrich), 108,5 mM ethanol (Carlo-Erba), with or without 10 μM Fe(II)-20 μM DETAPAC complexes (Sigma). Blanks without ethanol (flasks containing cells and POBN, +/− Fe(II)-DETAPAC in PBS) and blanks without cells (flasks only containing the intoxication medium, i.e. POBN, ethanol, +/− Fe(II)-DETAPAC in PBS), were tested in the same conditions for each incubation time.

The metal chelator DETAPAC was chosen because of its limited cellular toxicity, only observed for high concentrations, as opposed to other chelating agents such as the nitrilotriacetic acid, usually used in metal complexation in cells but described as cytotoxic and carcinogenic.[21,22] Moreover, despite limited cellular penetration, an intracellular accumulation of this chelate was shown by different authors who detected ^{14}C-chelates within the cell.[23,24]

After exposure, aliquots of the medium were immediately removed and analysed in the spectrometer. Adherent cells were rinced with buffer, harvested mechanically with a cell scraper, centrifuged, and the pellet was analysed spectrometrically in a flat cell, in the same conditions as for the medium.

EPR Experiments

The EPR spectra were recorded at room temperature, using an EPR spectrometer (BRUKER E300) at 100 KHz modulation. The microwave power was maintained at 20 mW, to avoid saturation and the modulation amplitude was set at 1.6 G. The other instrument settings were as follows: time constant, 200 msec; scan range, 100 G; sweep time, 30 min, unless otherwise noted. The extracellular medium was analysed in 20 μl calibrated micropipettes and the cell pellets in a flat quartz cell, immediately after sampling.

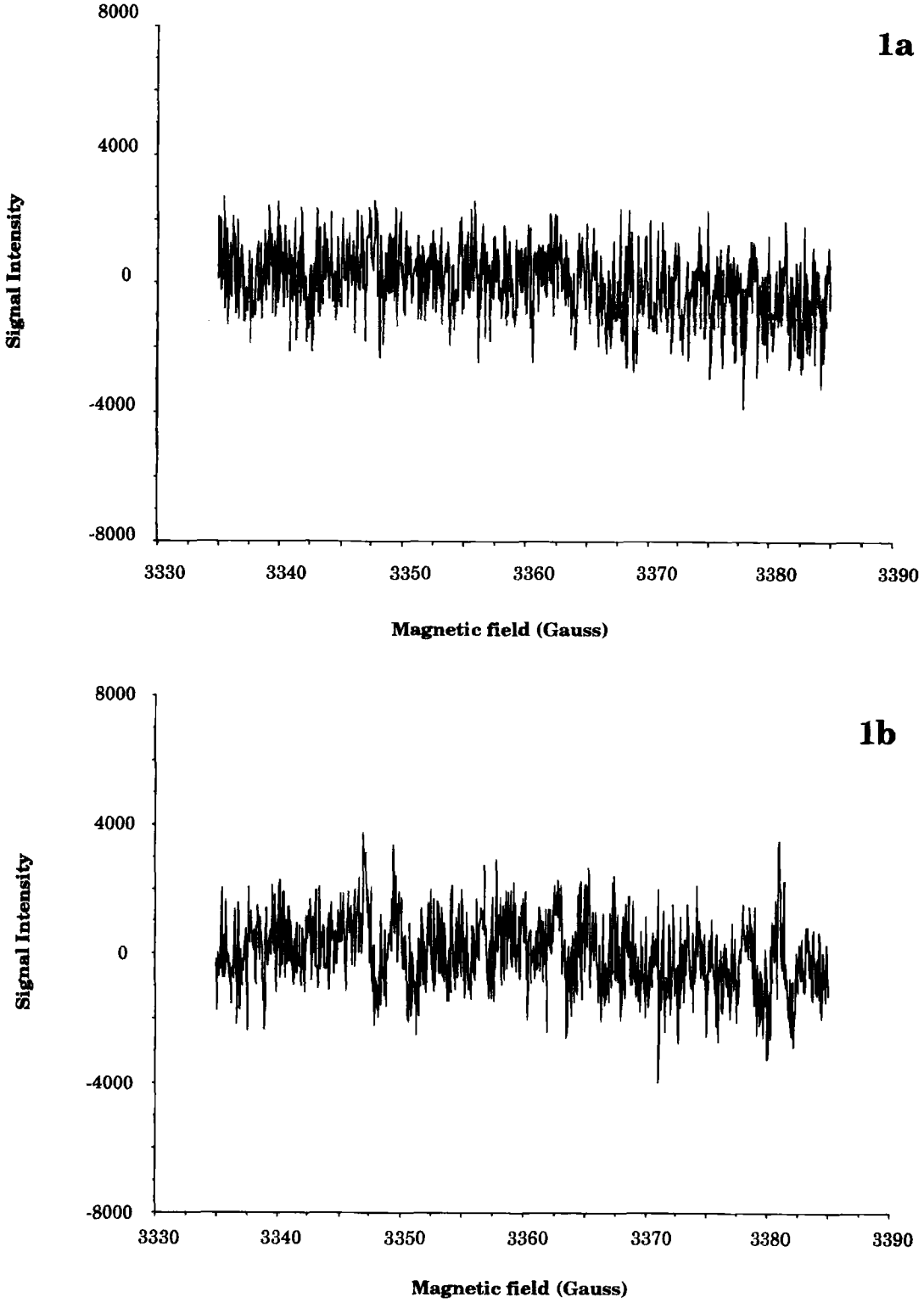

FIGURE 1 ESR spectra obtained with different controls. Spectrum (**1a**) was obtained when cells (astrocytes or C6) were incubated in the absence of ethanol and spectrum (**1b**) was obtained in the absence of cells, with our complete intoxication medium i.e. 50 mM POBN, 10 μM Fe (II)- 20 μM DETAPAC and 108.5 mM ethanol in PBS. The spectra were registered at room temperature and the instrument settings were as described in the Method section. ESR spectra were accumulated during 10 scans of 84 sec. each, using a gain amplitude of 6.3×10^4.

RESULTS

Cell Viability

Cell viability ranged from 89 to 93% in controls, and from 79 to 87% in astrocytes and C6 cells exposed to 108.5 mM alcohol, all values not statistically different.

Control Experiments

Controls without ethanol

No signal was detected when the cells were incubated without ethanol, at any time of incubation, even in the presence of the iron-DETAPAC complex (Fig. 1a).

Controls without cells

In systems only containing the complete intoxication medium (i.e. PBS containing POBN, Fe(II)-DETAPAC and ethanol), a very weak signal was registered, whose hyperfine splitting constants were identical to those measured for the α-hydroxyethyl radical. This signal, almost undetectable at the beginning of the incubation, increased slightly and then remained stable during the further 120 min of experimentation (Fig. 1b).

Detection of Radicalar Species after Ethanol Oxidation by a Fenton System

It has already been demonstrated that hydroxyl radicals generated during a Fenton reaction interact with ethanol to produce α-hydroxyethyl free radicals.[25] The addition of 20 μM hydrogen peroxide to a mixture containing 10 μM Fe(II)-20 μM DETAPAC complex, 108.5 mM ethanol and 50 mM POBN in PBS pH 7.4 gave rise in our experimental conditions to a spin-adduct showing hyperfine splitting constants of a_N = 15.63 G and a_H^β = 2.60 G (see Fig. 2). Moreover, in order to clearly identify this spin-adduct, the same

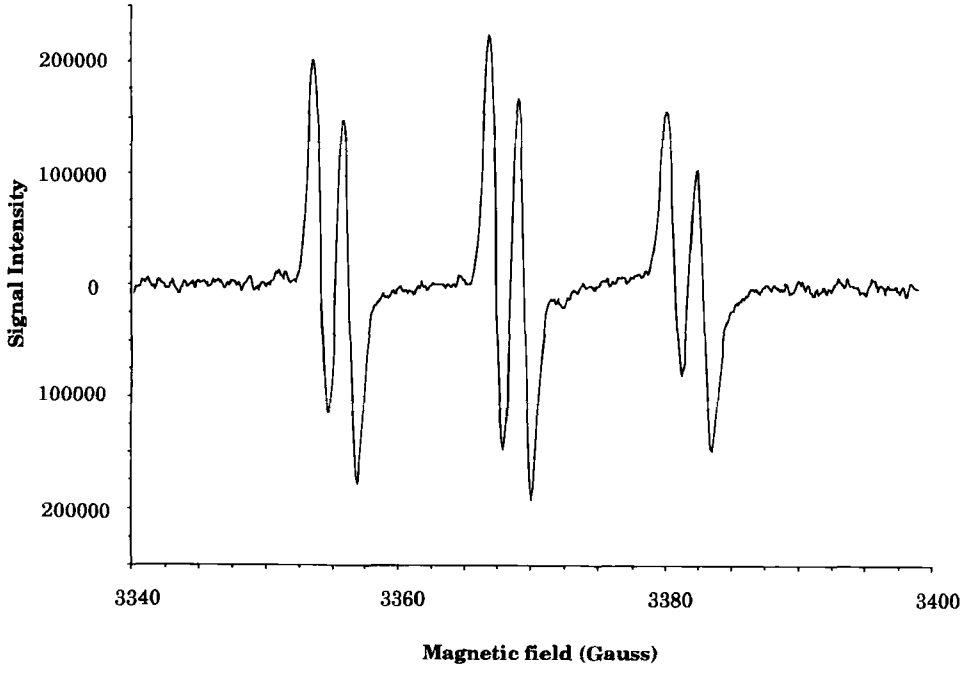

FIGURE 2 ESR spectrum obtained after addition of ethanol to a Fenton system. The mixture contained 10 μM $FeSO_4$, 20 μM DETAPAC, 50 mM POBN, 108.5 mM EtOH in PBS pH 7.4. The reaction was initiated by the addition of 50 μM H_2O_2. The spectrum was registered at room temperature, 20°C. The instrument settings were as described in the Method section and the spectrum was registered using a gain amplitude of 5×10^4 (single scan).

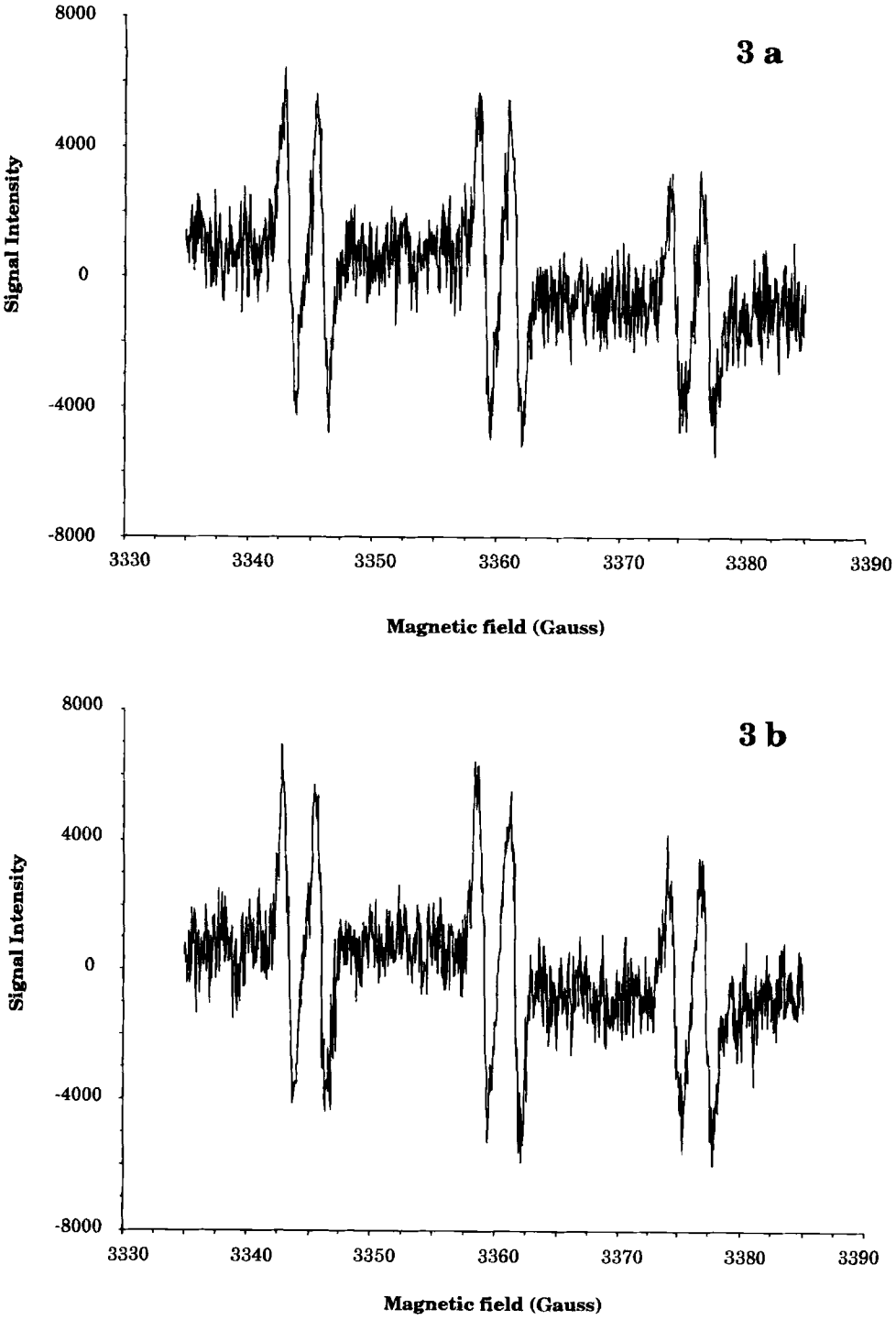

FIGURE 3 POBN-adduct formed after addition of ethanol in the intoxication medium of astrocytes (**a**) and C6 glioma cells (**b**). Complete intoxication mixture contained 10 μM FeSO4, 20 μM DETAPAC, 50 mM POBN and 108.5 mM EtOH in PBS pH 7.4. The final volume was 2.5 ml and the flasks were incubated one hour at 35°C. The spectra were registered at room temperature. The instrument settings were as described in the Method section and the ESR spectra were accumulated during 10 scans of 84 sec. each, using a gain amplitude of 6.4 10^4.

experiment was performed using ^{13}C-labelled ethanol. The typical 12-line signal obtained in such conditions confirmed the trapping of a CH3°CHOH radical (Fig. 4a).

Detection of Radicalar Species after Astrocyte Exposure to Ethanol

Cultured cells were separated by centrifugation from the intoxication medium (108.5 mM ethanol, 50 mM POBN +/– Fe(II)-DETAPAC in PBS pH 7.4) after being incubated from 15 to 120 min in this medium.

For each incubation period, the EPR signal was found in the extracellular medium, and never in the cell pellet. The signal intensity increased when the iron-DETAPAC complex was added to the intoxication medium (see Fig. 3a). The hyperfine splitting constants (a_N = 15.65 G and a_H^β = 2.63 G) were consistent with the trapping of a carbon-centered free radical.

These values were comparable to those obtained after ethanol oxidation by the Fenton system in our experimental conditions (see above) and also comparable to those quoted in the literature after photolysis or sonolysis of ethanol in water (a_N = 15,60 G and a_H^β = 2.65 G).[26] The signal therefore corresponded to the POBN adduct of the α-hydroxyethyl free radical. This was confirmed as for the Fenton reaction, by using ^{13}C-labelled ethanol, and obtaining a typical 12-line signal which identified a CH3°CHOH-POBN adduct (Fig. 4b).

The kinetic of the α-hydroxyethyl free radical production was studied during 120 minutes of incubation. Results were expressed according to the ESR signal intensity and to the protein concentration in the cell culture. This production was initially weak after 15 minutes of incubation and then progressively increased (Fig. 5).

Detection of Radicalar Species after Astrocytic C6 Glioma Cells Exposure to Ethanol

Cultured cells were also separated by centrifugation from the intoxication medium (108.5 mM ethanol, 50 mM POBN +/– Fe(II)-DETAPAC in PBS pH 7.4) after being incubated from 15 to 120 min in this medium.

The EPR signal was again observed only in the extracellular medium and never in the cell pellet (see Fig. 3b). The intensity of the signal was also increased in presence of the iron-DETAPAC complex in the same proportion as for the astrocyte experiments.

However, the signal was less intense in the case of exposure of C6 cells compared to astrocytes for a given protein concentration. Moreover, the kinetics of the radical production were also different from those observed after exposure of the astrocytes since the free radical formation remained weak and stable during the 120 minutes of intoxication (Fig. 5).

Finally, the hyperfine splitting constants were similar to those obtained after exposure of the astrocytes to ethanol and corresponded thus to the α-hydroxyethyl radical (a_N = 15.60 G and a_H^β = 2.65 G).

As for astrocyte experiments, incubation of C6 glioma cells with ^{13}C-labelled ethanol gave rise to a 12-line spectrum confirming the formation of an α-hydroxyethyl radical (Fig. 4b).

DISCUSSION

Astrocyte integrity is considered to be important for normal growth and functioning of neurons. Any impairment affecting these cells has therefore an effect on the functioning of the whole brain. In this context, recent studies have demonstrated the presence of cytochrome P-450IIE1 and NADPH cytochrome P-450 reductase in astrocytes, and their possible induction by ethanol.[11,12] These two enzymes, located in the endoplasmic reticulum, can metabolize ethanol via a radicalar pathway involving the formation of reactive oxygen species, followed by that of the α-hydroxyethyl radical. This fact could thus bring new insight for the comprehension of brain ethanol-induced toxicity. However, the formation of this radical has never

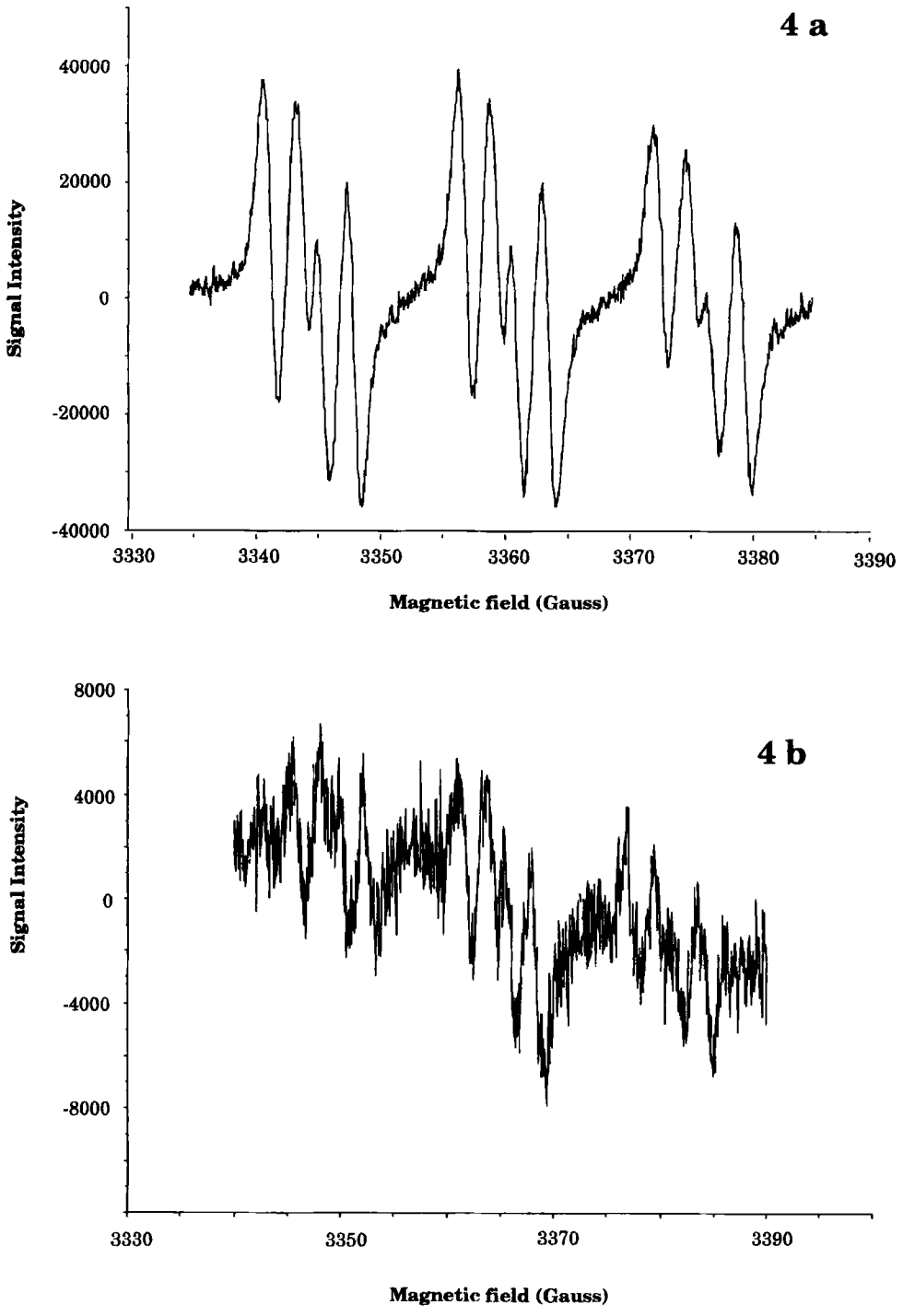

FIGURE 4 POBN-adduct formed after addition of (1-^{13}C)- labelled ethanol to our Fenton system (**a**) or in the intoxication medium of astrocytes and C6 glioma cells (**b**). Complete intoxication mixture contained 10 μM FeSO4, 20 μM DETAPAC, 50 mM POBN, 108.5 mM EtOH in PBS, pH 7.4. The final volume was 2.5 ml and the flasks were incubated one hour at 35°C. The spectra were registered at room temperature and the instrument setting were as described in the Method section. Spectrum (**a**) was registered using a gain amplitude of 5×10^4 (single scan) and spectrum (**b**) results from the accumulation of 10 scans of 84 sec. each, using a gain amplitude of 6.4 10^4.

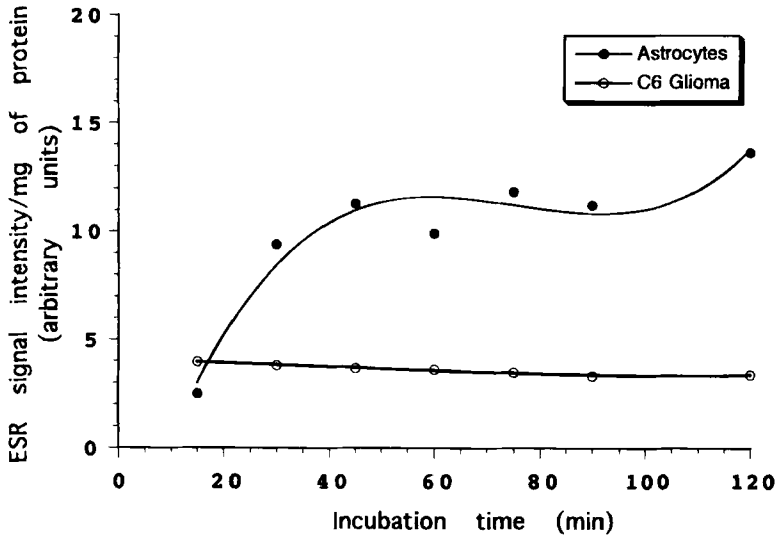

FIGURE 5 Kinetics of the α-hydroxyethyl radical formation during astrocytes (–●–) and C6 glioma cells (–○–) intoxication by ethanol. The ESR signal intensity was calculated in both cases for a given protein concentration expressed in mg and for each incubation time. Experimental conditions were as described in the Method section.

been demonstrated experimentally using EPR, before the present study in isolated cells in culture.

Cell cultures have indeed become an important tool in research, and particularly in toxicology. The reactivity of different types of isolated cells can be compared without having to take into account side-effects such as undernutrition following prolonged and heavy animal exposures.[27] Culture aggregate or monolayer culture systems have been successfully used to elucidate several neurotoxic cellular actions of CNS xenobiotics.[28]

EPR using a spin-trapping technique has also greatly contributed to the study of the mechanisms of toxicity by allowing direct identification of free-radicals formed during the metabolism of several exogenous compounds, such as acetaminophen, halogenated solvents, paraquat.[29,30,31] However, the use of spin-traps in biological systems could be a potential source of problems and artefacts. For example, the choice of the spin-trap molecule is very important because most of the spin-traps used are toxic for the cell or interact with enzymes such as cytochrome P-450 and NADPH-cytochrome P-450 reductase, both involved in the metabolism of ethanol by microsomal systems via a free radical pathway. DMPO does not inhibit cytochrome P-450 and NADPH cytochrome P-450 reductase. Unfortunately, it causes a significant loss in cell viability (50%) in our conditions of intoxication.[32] Moreover, chelated iron in phosphate buffer produces pseudo-radical adducts in the presence of this spin-trap.

In this study, we used the α-(4 pyridyl-1-oxyde)N-tert butyl nitrone (POBN) because of its type II binding spectrum to cytochrome P-450, corresponding to binding to the haem iron of the cytochrome moiety, so excluding an interaction with the substrate binding site, as it is observed for PBN, another commonly used spin-trap.[33] Moreover, POBN is not toxic to isolated cells as demonstrated by the evolutions of intracellular LDH and glutathione following POBN addition.[33] Finally, POBN is considered to be the most useful spin-trap in biological systems. In this preliminary study combining the advantages of cell cultures with EPR spectroscopy, cultured astrocytes and astrocytic-like cells (C6 glioma cells) were shown to be able to produce free radicals deriving from ethanol as confirmed by the

use of ^{13}C-labelled ethanol. Although the same type of free radical was trapped after exposure of the two kinds of cells, free radical production was less important in the cell lines. Astrocytes appeared to be more sensitive than C6 glioma cells to free radical formation.

Detection of the trapped α-hydroxyethyl radical in the extra-cellular medium only does not mean that it was not generated within the cells. Indeed, two different kinds of blanks were performed, in order to confirm the cellular origin of the trapped radicals.

Using the same experimental conditions throughout, we first carried out experiments with cells in the absence of ethanol. Under such conditions, no EPR signal was registered at any time of incubation (Fig. 1a), confirming that the trapped radicals derived directly from the added alcohol.

Secondly, experiments were then conducted in the presence of the complete intoxication medium (i.e. PBS containing 50 mM POBN, 10 μM Fe(II)- 20 μM DETAPAC and 108.5 mM ethanol) but in the absence of cells. A very weak signal was registered, whose hyperfine splitting constants were identical to those measured for the α-hydroxyethyl radical (Fig. 1b). This signal, almost undetectable at the beginning of the incubation, increased only slightly and then remained stable during the further 30 to 120 minutes of the experiments.

This signal could correspond to radical production by a Fenton-type reaction in the absence of hydrogen peroxide, as described by Reinke *et al.*.[34] This α-hydroxyethyl radical generation was assigned to the auto-oxidation of Fe (II) by the phosphates present in the buffer (PBS) and in the presence of ethanol. In their experiments, the highest EPR signal was registered for a 100 μM concentration in ferrous salt. Using Reinke's conditions of experimentation, we reproduced such a signal. However, when applied to our experimental conditions (i.e. an iron concentration 10 times lower than that tested by Reinke and the use of chelated iron instead of the free salt), the signal had a considerably lower intensity (Fig. 1b). Moreover, when compared with the signal obtained in the presence of cells (Fig. 3a and 3b), its intensity was also significantly lower.

Finally, concerning the mechanism of formation of the α-hydroxyethyl radical, two hypotheses could be discussed. On one hand, the α-hydroxyethyl radical which was only detected extracellularly could be due to the reaction of the Fe-DETAPAC complex with traces of superoxide and/or hydrogen peroxide, leading first to the formation of a hydroxyl radical and then, in the presence of ethanol, to the generation of the α-hydroxyethyl radical. Indeed, several authors have shown that reactive oxygen species produced intracellularly can leave the cell by passive diffusion across the cell membrane or via the membrane anion channel.[35,36,37] Nevertheless if this was true, the incubation of cells in the intoxication medium in the absence of ethanol (i.e. buffer only containing POBN and the Fe-DETAPAC complex) would have produced OH° and / or $O_2^{-\circ}$-POBN adducts as shown by different authors.[19] In our experiments, no signal was registered in such a condition, a result which does not favour the above hypothesis. However, the results obtained in presence of cells and ethanol could also means that the OH° and/or O2$^{-\circ}$ radicals produced during the metabolism of ethanol, and not in its absence, can be excreted and react with ethanol in the extracellular medium.

On the other hand, the α-hydroxyethyl radical could also be formed within the cells and then excreted in the extracellular medium where it is only identified because of the particular conditions of the intracellular medium. Indeed, the absence of EPR signal detection in the cell pellet could be due to the rapid reduction of the trapped radical into undetectable hydroxylamine by various molecules present inside the cell such as ascorbate, thiol functions, enzymes, all molecules not present in the extracellular medium in our experimental conditions.

Finally, experiments conducted without astrocytes or without ethanol, clearly demonstrated

that the presence of both cells and ethanol is necessary to obtain the α-hydroxyethyl radical.

We also demonstrated that the addition of the iron-DETAPAC complex enhanced the production of the free radicals, reproducing an *in vivo* effect which could be important in terms of cell sensitization to ethanol exposure.

Indeed, several authors have reported that the presence of transition metal traces enhanced the formation of radicalar species in the liver and the brain after ethanol intoxication[3,8,13] and furthermore that an acute ethanol load increased the non-haem iron content in the liver and the cerebellum,[38] both facts leading to an increased free-radical formation.

In conclusion, the α-hydroxyethyl radical was identified after ethanol exposure of astrocytes and C6 glioma cells in culture. After exposure of astrocytes, the signal increased progressively during the period of incubation from 15 to 120 min, a result not observed with C6 glioma cells. Moreover, after exposure the signal obtained with the latter cells was less intense compared to astrocytes for a given protein concentration. Astrocytes therefore appeared to be more sensitive to free radical formation than C6 glioma cells.

Acknowledgments

This work was supported by the "Institut de Recherches Scientifiques sur les Boissons". (I.R.E.B., Paris). We greatly thank Jean Jimenez and Philippe Belmont for their helpful advice and their disposal, Mrs F. Hemming (Laboratoire de Neurobiologie du développement, CERMO, Université Joseph FOURIER, GRENOBLE) for helpful suggestions concerning the manuscript and Pr Feuerstein for giving us the glial cell lines.

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