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FREE RADICAL METABOLISM OF ALCOHOLS BY RAT LIVER MICROSOMES

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By using e.s.r. spectroscopy coupled with the spin trapping technique we have detected the formation of free radical intermediates by rat liver microsomes incubated with either ethanol, 2-propanol or 2-butanol in the presence of a NADPH regenerating system and 4-pyridyl-1-oxide-t-butyl nitrone (4-POBN) as spin trap. The e.s.r. spectra have been identified as due to the hydroxyalkyl free radical adducts of 4-POBN.

The free radical formation depends upon the activity of the microsomal monoxygenase system and is blocked by omitting $NADP^+$ from the incubation mixture, by anaerobic incubation or by enzyme denaturation. The involvement of hydroxyl radicals ($OH^{\cdot}$) produced through a Fenton-type reaction from endogenously formed hydrogen peroxide is suggested by the opposite effects exerted on the e.s.r. signal intensity by azide and catalase. Consistently, iron chelation by desferrioxamine inhibits the free radical formation, while the supplementation of EDTA-iron increases it by several fold. Inhibitors of cytochrome P_{450}-dependent monoxygenase system reduce to various extents the production of free radical intermediates suggesting that reactive oxygen species might be formed at the active site of cytochrome P_{450} where they react with alkyl alcohol molecules.

The data presented support the hypothesis that free radical species are generated during the microsomal metabolism of alcohols and suggest the possibility that ethanol-derived radicals might play a role in the pathogenesis of the liver lesions consequent upon alcoholic abuse.

KEY WORDS: Free radicals, spin trapping, ethanol, alcohol metabolism.

INTRODUCTION

The ability of rat liver microsomes to oxidize ethanol was first described by Orme-Johnson and Ziegler[1] and characterized as independent from contaminations by catalase or alcohol dehydrogenase.[2] Subsequent studies have shown that liver microsomes are also capable of oxidizing other aliphatic alcohols such as propanol and butanol to the corresponding aldehydes.[3] This metabolic pathway requires the presence of NADPH and oxygen, and seems to depend upon the activity of cytochrome P_{450}-dependent monoxygenase enzymes.[4] Studies with reconstituted systems clearly indicate that, differently from other monoxygenase activities, ethanol oxidation requires hydroxyl radicals ($OH^{\cdot}$).[5,6] The reaction is, in fact, inhibited in a competitive fashion by $OH^{\cdot}$ scavengers, while the addition of iron-EDTA stimulates it by several fold.[5-7] Therefore, it has been suggested that hydroxyl radicals can be generated in a Fenton-type reaction between H_2O_2, produced by the microsomal

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electron transport system and ferrous iron.[5,6] Similar results have also been obtained using liver microsomes incubated with ethanol, isopropanol, 1-butanol and 2-butanol.[8-10] In these studies, the catalase inhibitor azide stimulates alcohol oxidation,[8-10] while iron chelation by desferrioxamine greatly reduces it.[9,11]

If an OH˙ radical-dependent pathway is involved in the oxidation of alcohols, free radical species are likely to be produced as transient intermediates. In the present work, we have investigated by using the electron spin resonance (e.s.r.) spin trapping technique the production of free radical metabolites taking place in liver microsomes incubated with ethanol, 2-propanol and 2-butanol. Short-lived free radicals can be demonstrated in biological systems by allowing them to interact with spin traps possessing a nitrone functional group to form comparatively long-lived nitroxide radical adducts that can be extracted and analyzed by e.s.r. spectroscopy.[12]

MATERIALS AND METHODS

Rat liver microsomes were prepared from male Wistar rats (200–250 g body wt.) as described by Slater and Sawyer,[13] except that the livers were perfused with ice-cold saline to remove blood prior to homogenization.

Microsomes (approx. 2 mg protein/ml) were incubatd at 37°C in 25 ml Erlenmeyer flasks closed with screw caps. The incubation mixture consisted of three parts of 0.1 M phosphate buffer pH 7.4, two parts of 0.15 M KCl, 10 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.5 mM $NADP^+$, 25 mM 4-pyridyl-1-oxide-t-butyl nitrone (4-POBN) in a volume of 1.9 ml. The alcohols (20 mM final concentrations) and various agents were dissolved in water and added to the basic reaction mixture to made up the final volume of 2 ml. Carbon monoxide was bubbled for 1 min through the microsomal suspension before alcohol addition. The reaction was initiated by adding 0.7 units glucose-6-phosphate dehydrogenase dissolved in a small volume of buffer.

The incubation was terminated after 30 min by extracting the spin adduct with 1 ml of chloroform–methanol (2:1 v/v) mixture as previously described[14] and the organic phase was used for the e.s.r. analysis.

A Bruker 200D SRC spectrometer fitted with a variable temperature control was used. The instruments settings were as follows: microwave power 10 mW; modulation frequency 100 MHz; modulation amplitude 1 G; scan width 100 G; sample temperature −50°C.

The spin trap 4-pyridyl-1-oxide-t-butyl nitrone (4-POBN) was obtained from Aldrich–Europe (Beerse, Belgium), p-chloro-mercuribenzoate (pCMB), metyrapone and azide from Sigma Chemical Co. (St. Louis, USA). $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and catalase were purchased from Boehringer Biochemia (Mannheim, F.R.G.). Ethanol labelled with [^{13}C] isotope on both the carbon atoms was obtained from Merck Sharp & Dohme Isotopes (Montreal, Canada). Desferrioxamine was kindly supplied by Ciba-Geigy (Basel, Switzerland). SKF 525A was a gift from Smith, Kline & French Ltd. (Welwyn Garden City, U.K.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.).

RESULTS

The incubation of liver microsomes with either 20 mM ethanol, 2-propanol or 2-butanol in the presence of a NADPH-regenerating system and 25 mM 4-POBN led to the

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formation of different e.s.r. spectra (Figure 1), the hyperfine splitting constants of which are reported in Table I. No signals were detectable when alcohols were omitted from the incubation mixture (Figure 1). Upon the addition of 20 mM [^{13}C] ethanol, a further splitting in the e.s.r. spectrum was observed (Figure 1), giving a clear

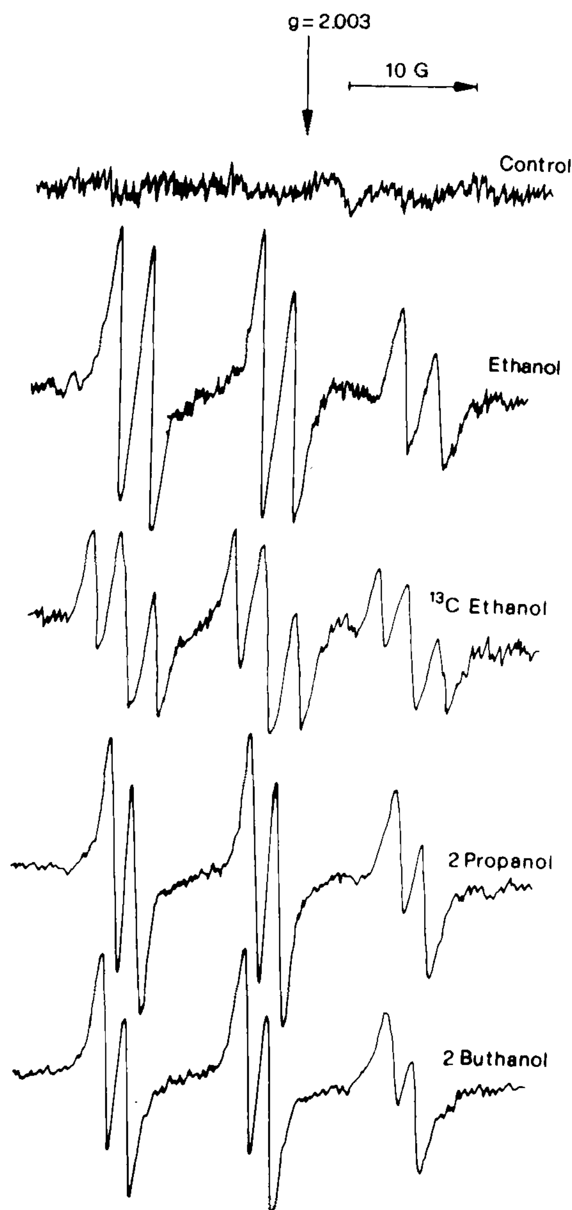

FIGURE 1 e.s.r. spectra of the 4-POBN free radical adducts formed in rat liver microsomes incubated with 20 mM ethanol, [^{13}C]-labelled ethanol, 2-propanol and 2-butanol. The upper trace refers to control preparations incubated without alcohols. The recorder gain was 10^6 for the upper three traces and $10^5 \times 5$ bottom two traces.

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TABLE I
Hyperfine splitting constants of the 4-POBN spin adducts produced from different alcohols in liver microsomes or during a Fenton-type reaction

	Microsomal incubation		Fenton reaction	
	aN	aH	aN	aH
Ethanol	14.97	3.48	14.97	3.50
[^{13}C] Ethanol	14.97	3.47	–	–
Methanol	–	–	14.78	3.56
2-Propanol	14.98	2.67	15.13	2.92
2-Butanol	15.10	2.56	15.18	2.64

The values are expressed in Gauss and are means of 3–5 different measurements.

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indication that the radical trapped in the presence of ethanol was derived from this alcohol molecule.

The hyperfine splitting constants of the nitroxide adducts formed by microsomes incubated with ethanol, 2-propanol and 2-butanol were similar to those produced by the same compounds when chemically activated to free radicals by a Fenton-type reaction (Table I). This finding, along with the detection of the [^{13}C] hyperfine splitting, indicated that the free radical species derived from ethanol can be identified as the hydroxyethyl radical. On the same basis the spin adducts produced by 2-propanol and 2-butanol were ascribed to, respectively, 2-hydroxypropyl and 2-hydroxybutyl free radicals.

As shown in Figure 2, the intensity of the e.s.r. signals produced by microsomes incubated with either 20 mM methanol, ethanol, 2-propanol or 2-butanol varied concomitantly with the increase in the molecular weight of alcohols; methanol addition, however, resulted in only a small and unresolved e.s.r. spectrum.

The free radical activation of aliphatic alcohols strictly depended upon the activity of the microsomal monoxygenase system since the omission of $NADP^+$ or the denaturation of the enzymes completely blocked it (data not shown). Likewise, the incubation of liver microsomes under a nitrogen atmosphere inhibited by more than 90% the radical formation (data not shown).

Concerning the mechanisms involved in the generation of the free radical species the possible role of $OH^{\cdot}$, resulting from the degradation of H_2O_2 in the presence of iron, has been investigated.

As shown in Table II, the intensity of the e.s.r. signals due to ethanol, 2-propanol and 2-butanol were increased by approx. 2–3-fold in microsomes pretreated with azide, in order to inhibit the disposal of endogenous H_2O_2. On the contrary, supplementing catalase reduced on various extent the amount of free radical trapped (Table II). The critical role played by iron in catalyzing alcohol activation was evident from the increase in the e.s.r. signal intensities resulting from the addition of 0.05 mM iron chelated with EDTA. Conversely, desferrioxamine which abolished the formation of $OH^{\cdot}$ radicals by complexing iron ions,[11] decreased by approx. 80% the e.s.r. signal intensities (Table II).

The production of alcohol-derived free radicals was also affected by inhibitors of the mixed function oxidase system, such as SKF 525A, metyrapone, carbon monoxide and p-chloro-mercuribenzoate (pCMB). Different alcohols showed a variable susceptibility to the effects of "classical" cytochrome P_{450} inhibitors; for instance, SKF 525A lowered by 50% and 64% the e.s.r. signals due to 2-propanol and 2-butanol, respectively, but only by 30% that of ethanol (Table II). Metyrapone, instead, caused comparable effects on the activation of the three alcohols, depressing them to about

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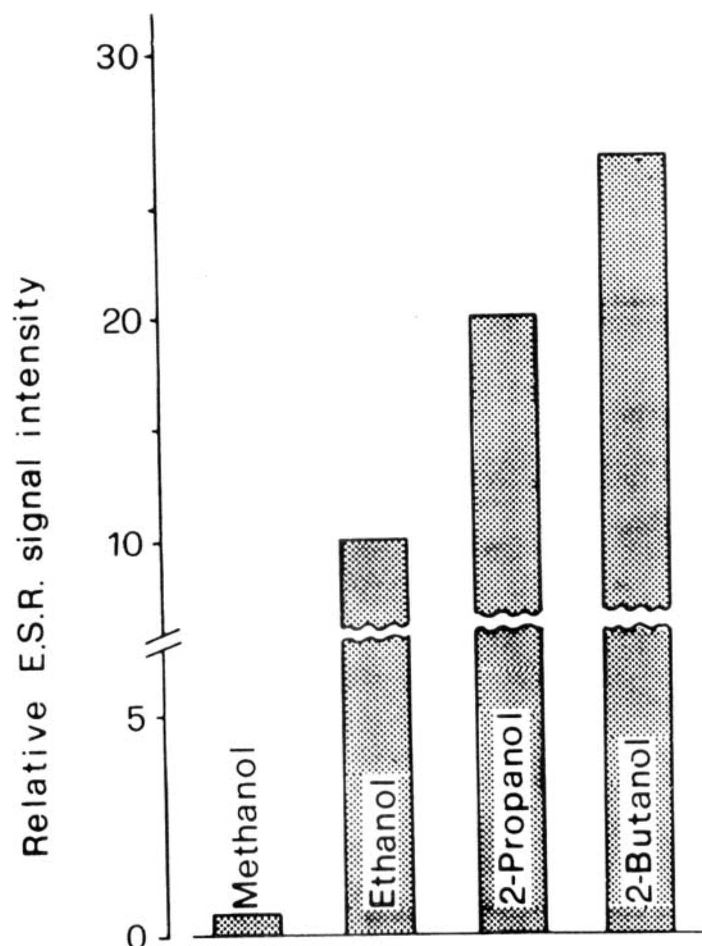

FIGURE 2 Intensity of the e.s.r. signals produced in liver microsomes incubated 30 min at 37°C with 20 mM of either methanol, ethanol, 2-propanol and 2-butanol.

TABLE II
Effect of various treatments that influence the formation of alcohol-derived free radical by liver microsomes

	% of the controls		
	Ethanol	2-Propanol	2-Butanol
Azide 1 mM	269	300	173
Catalase 500 U	43	55	36
$FeCl_3$ 0.05 mM-EDTA 0.1 mM	339	650	376
Desferrioxamine 0.25 mM	18	20	17
SKF 525A 1 mM	70	50	36
Metyrapone 0.5 mM	56	68	67
Carbon monoxide	96	90	64
pCMB 0.1 mM	49	37	42

The values are expressed as a percent of the e.s.r. signal intensities in the respective control preparations incubated with either 20 mM ethanol, 2-propanol and 2-butanol. The results are means of two different determinations.

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60–70% of control values (Table II). Carbon monoxide addition did not appreciably interfere with the free radical formation from ethanol or 2-propanol, while it inhibited by approx. 40% that from 2-butanol. The thiol reagent pCMB, used in low concentration to block the electron flow to cytochrome P_{450}, also reduced by about 50–60% the production of e.s.r. signals from all three alcohols.

DISCUSSION

Several studies have shown that alcohol oxidation by liver microsomes is mediated by reactive oxygen species, most likely the hydroxyl free radical ($OH^{\cdot}$).[5–11] By using the combination of electron spin resonance (e.s.r.) spectroscopy and the spin trapping technique we have demonstrated that free radical species are produced during the microsomal metabolism of ethanol, 2-propanol and 2-butanol.

The hyperfine splittings of the free radical adducts obtained in the microsomes were very close to the values of the 4-POBN adducts produced by a Fenton-type reaction in the presence of the same alcohols. It is known that hydroxyalkyl radicals are generated by the interaction of $OH^{\cdot}$ with alcohols, therefore the similarities in the spectral features allow the identification of hydroxyethyl, 2-hydroxypropyl and 2-hydroxybutyl free radicals as the reactive intermediates originating from ethanol, 2-propanol and 2-butanol, respectively. The trapping of carbon centered free radicals from alcohol molecules is supported by the detection of [^{13}C] hyperfine splitting when ethanol labelled with this isotope has been used.

Acetaldehyde, acetone and 2-butanone have been detected as the main products of microsomal oxidation of ethanol, 2-propanol and 2-butanol, respectively.[7–10] It can be postulated that the hydroxy alkyl radicals might react with oxygen to make the corresponding peroxyl radicals, which on their term could abstract hydrogen atoms from neighbouring molecules to form the hydroperoxy-derivatives. Breakdown of these latter compounds through alkoxyl radicals would then originate the corresponding aldehydes or ketones.

Concerning the mechanisms responsible for alcohol activation, spin trapping results are in agreement with the studies performed by the group of Cederbaum[6–10] indicating the involvement of an oxygen radical-dependent pathway. The formation of the hydroxyalkyl radicals requires, in fact, the presence of H_2O_2 and iron and is strongly inhibited by desferrioxamine.

This latter compound may not only act as iron chelator but also as a scavenger for $OH^{\cdot}$ and superoxide ion, as recently reported.[15,16] Evidence in the literature suggests that two pathways are possibly responsible for the oxidation of both ethanol and butanol by liver microsomes. One pathway involves the interaction of alcohols with $OH^{\cdot}$ generated during microsomal electron transfer, whereas the other appears to depend upon a cytochrome P_{450} catalyzed reaction.[10,17]

The effect exerted by some inhibitors of the mixed function oxidase system suggests that the activity of cytochrome P_{450} might be, at least in part, involved in catalyzing the free radical formation. Nonetheless the various alcohols behave quite differently toward the effects of cytochrome P_{450} inhibitors making difficult to establish the exact role played by the haemoprotein. It is possible that perferryl-cytochrome P_{450} complexes, which have reactivities comparable to that of $OH^{\cdot}$, might take part in the free radical activation process. It is noteworthy, that methanol having a rate constant for $OH^{\cdot}$ not too different from that of ethanol or 2-propanol[15] does not produce detect-

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able amounts of spin adduct in microsomal incubations, whereas it does in a chemical system with Fenton reagents. Methanol, however, is not a substrate for the monoxygenase system metabolizing ethanol.[2] Almost the opposite has been observed with 2-butanol, a good substrate for cytochrome P_{450},[10] but not as reactive with $OH^{\cdot}$ as ethanol or 2-propanol.[15]

The production of ethanol free radical intermediates could provide a reason for the stimulation of lipid peroxidation and for the lowering of hepatic glutathione often observed in the liver of animals intoxicated with ethanol[18,19] as well as in the hepatic biopsies obtained from alcoholic patients.[20] Thus, a free radical mediated pathogenesis can be postulated as a cause of some of the liver damages consequent to the abuse of alcoholic beverages.

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