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ROLE OF ETHANOL-INDUCIBLE CYTOCHROME P450 (P450IIE1) IN CATALYSING THE FREE RADICAL ACTIVATION OF ALIPHATIC ALCOHOLS

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Abstract—Incubation of rat liver microsomes with 1-propanol and 1-butanol in the presence of NADPH and of the spin trapping agent 4-pyridyl-1-oxide-*t*-butyl nitrone (4-POBN) allowed the detection of free radical intermediates tentatively identified as 1-hydroxypropyl and 1-hydroxybutyl radical, respectively. Microsomes isolated from rats treated chronically with ethanol (EtOH) or with the combination of starvation and acetone treatment (SA), exhibited a two-fold increase in the ESR signal intensity as compared to untreated controls, whereas no increase was observed in phenobarbital-induced (PB) microsomes. Consistently, in reconstituted membrane vesicles, ethanol-inducible cytochrome P450IIE1 was twice as active as phenobarbital-inducible P450IIB1 in producing 1-butanol free radicals. In the microsomal preparations from EtOH and SA pretreated rats the addition of antibodies against cytochrome P450IIE1, but not of preimmune IgGs, lowered the ESR signal of 1-butanol radicals by more than 50%. The same antibodies decreased the free radical production by untreated microsomes by 35–40%, but were ineffective on microsomes from PB-treated animals. This indicated that cytochrome P450IIE1 is the major enzyme responsible for the free radical activation of alcohols in control and ethanol-fed rats. The generation of 1-hydroxybutyl radicals by EtOH microsomes was inhibited by 40, 48 and 68%, respectively, by the addition of isoniazid, tryptamine and octylamine, compounds known to specifically affect the NADPH oxidase activity of this isoenzyme. This effect was not due to the scavenging of the alcohol radical since none of these compounds affected the ESR signals originated from 1-butanol in a xanthine–xanthine oxidase system. When added to reconstituted membrane vesicles isoniazid, tryptamine and octylamine also decreased 1-butanol radical formation by P450IIE1 by 54, 38 and 66%, respectively. Such an inhibition corresponded to the effect exerted by the same compounds on O_2^- release from P450IIE1 containing vesicles. These results indicate that the capacity of cytochrome P450IIE1 to reduce oxygen is related to its ability to generate alcohol free radicals and suggest that ferric cytochrome P450–oxygen complex might act as oxidizing species toward alcohols.

The capacity of rat liver microsomes to oxidize ethanol and other aliphatic alcohols to the corresponding aldehydes was first documented by Orme-Johnsson and Ziegler [1]. This metabolic pathway required the presence of NADPH and oxygen and was found to be mediated upon the activity of cytochrome P450 dependent monooxygenase system [2]. Subsequent studies by the group of Cederbaum suggested the possibility that hydroxyl radicals ($OH^\cdot$), generated as a result of iron-catalysed decomposition of hydrogen peroxide, were involved in the metabolism of alcohols by liver microsomes [3–6]. Carbon centered free radicals are expected to be formed from the reaction of alcohols and $OH^\cdot$ and indeed we have detected the formation of 1-hydroxyethyl, 2-hydroxypropyl and 2-hydroxybutyl radicals by rat liver microsomes incubated with, respectively, ethanol, 2-propanol and 2-butanol [7, 8].

Recent results obtained in experiments using deuterium substitution showed, however, the occurrence of both an $OH^\cdot$-dependent and an $OH^\cdot$-independent mechanisms of ethanol oxidation [9]. Similar conclusion were also reached by investigating the role played by $OH^\cdot$ in the formation of 1-hydroxyethyl radical by liver microsomes [8].

A number of studies have shown that the ethanol-inducible form of cytochrome P450, known as cytochrome P450IIE1 is specifically active in catalysing the oxidation of ethanol as well as of other aliphatic alcohols [10–13]. Moreover, ethanol treatment of rats despite increasing the rate of ethanol metabolism by liver microsomes, did not stimulate the formation of $OH^\cdot$ radical as estimated by the metabolism of dimethylsulfoxide to formaldehyde [13]. This finding along with the observation that microsomes from ethanol-pretreated rats were able to produce hydroxyethyl free radicals even in the absence of an appreciable generation of $OH^\cdot$ species [8], led to the suggestion that cytochrome P450 might be responsible for the formation of free radical intermediates during the metabolism of aliphatic alcohols.

The aim of this work was to evaluate this hypothesis and to investigate the mechanisms by

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Table 1. Hyperfine splitting constants of the 4-POBN free radical adducts produced during the incubation of 1-propanol or 1-butanol with rat liver microsomes, reconstituted membrane vesicles containing cytochrome P450 or the xanthine–xanthine oxidase system

	Liver microsomes		Reconstituted vesicles		Xanthine–xanthine oxidase system	
	aN	aH	aN	aH	aN	aH
1-Propanol	13.65	3.27	—		13.67	3.29
1-Butanol	15.01	3.45	15.02	3.48	14.95	3.41

The spectral features are expressed in Gauss and were calculated on 5–7 different ESR spectra.

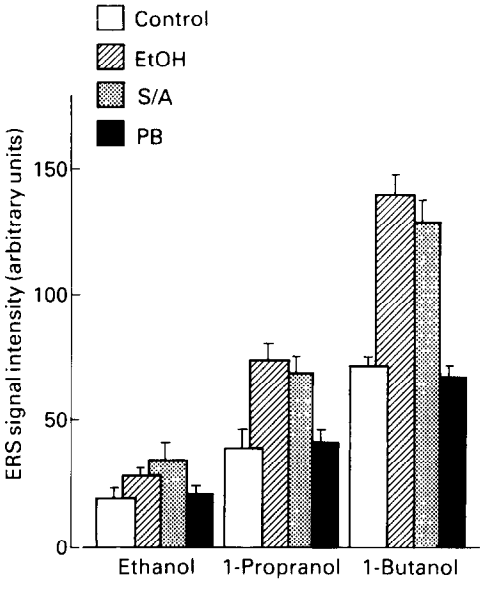

Fig. 1. Effect of chronic ethanol feeding (EtOH), starvation and acetone treatment (S/A) or phenobarbital administration (PB) on the spin trapping of alcohol-derived free radicals in rat liver microsomes. Microsomal fractions were incubated for 5 min at 37° with 20 mM of ethanol, 1-propanol and 1-butanol, respectively, as reported in Materials and Methods. The results are means of 3–5 different determinations ± SD.

which cytochrome P450, and in particular the ethanol-inducible form P450IIE1, was able to generate alcohol free radicals.

MATERIALS AND METHODS

NADPH, tryptamine, isoniazid, pargyline, xanthine oxidase and xanthine were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 1-Butanol, 1-propanol, *p*-nitrophenol, and the spin trap 4-pyridyl-1-oxide-*N*-*t*-butyl nitrone (4-POBN) were purchased from Aldrich Europe (Bersee, Belgium). Desferrioxamine methane-sulphonate (Desferal) was supplied by Ciba-Geigy (Basel, Switzerland). Anti-P450IIE1 immunoglobulins were prepared by ammonium sulphate precipitation from immunized rabbits as previously described [14].

Male Sprague–Dawley rats (150–170 g) were used for all the experiments. Chronic ethanol administration was performed for 20 days using the liquid diet of DeCarli and Lieber [15] and control rats were pair fed. Other rats were starved for 72 hr and treated with acetone (5 mL/kg body wt) given intragastrically as a 33% (v/v) solution in saline for the last 2 days as previously described [14]. Phenobarbital treatment (80 mg/kg) was performed i.p. for 3 days. All rats except the pair fed and the starved animals received food and water *ad lib.*

Liver microsomes were prepared by ultracentrifugation of 20% (w/v) homogenate in 10 mM Na/K-phosphate buffer, pH 7.4, containing 1.14% KCl and washed once before suspension in 50 mM K-phosphate buffer, pH 7.4 [14]. For the spin trapping experiments microsomal fractions (5 mg protein) were added to 1 mL (final volume) of an incubation mixture containing two parts of 0.1 M Na-phosphate buffer pH 7.4, three parts of 0.15 M KCl, 1 mg NADPH, 20 mM of either ethanol, 1-propanol or 1-butanol, 25 mM 4-POBN and 25 μM desferrioxamine and incubated for 5 min at 37° in 5 mL stoppered test tubes. The incubations were terminated by transferring the samples to 1.5 mL Eppendorf tubes containing 0.5 mL of a chloroform–methanol (2:1 v/v) mixture as previously reported [8]. The chloroform phase was separated by centrifugation and used for ESR analysis.

ESR spectra were recorded at room temperature using a Bruker D 200SRC spectrometer with the following instrument setting: microwave power 10 mW; modulation frequency 100 KHz; modulation amplitude 1 G; field scan 100 G.

Free radicals were also produced from alcohols by incubating for 30 min at 37° 1-propanol or 1-butanol (20 mM) with 1 mM xanthine, 0.05 units xanthine oxidase, 50 mM 4-POBN in 1 mL 50 mM Na-phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 50 μM $FeCl_3$. The spin adducts were then extracted with 0.5 mL chloroform–methanol mixture as described above.

Cytochromes P450IIE1 and P450IIB1 were prepared from liver microsomal fractions obtained from starved/acetone treated (S/A) and phenobarbital treated (PB) rats, respectively, according to Johansson and co-workers [14]. NADPH cytochrome P450 reductase was purified from liver microsomal fractions of starved/acetone treated rats [14]. Microsomal phospholipids were extracted from liver

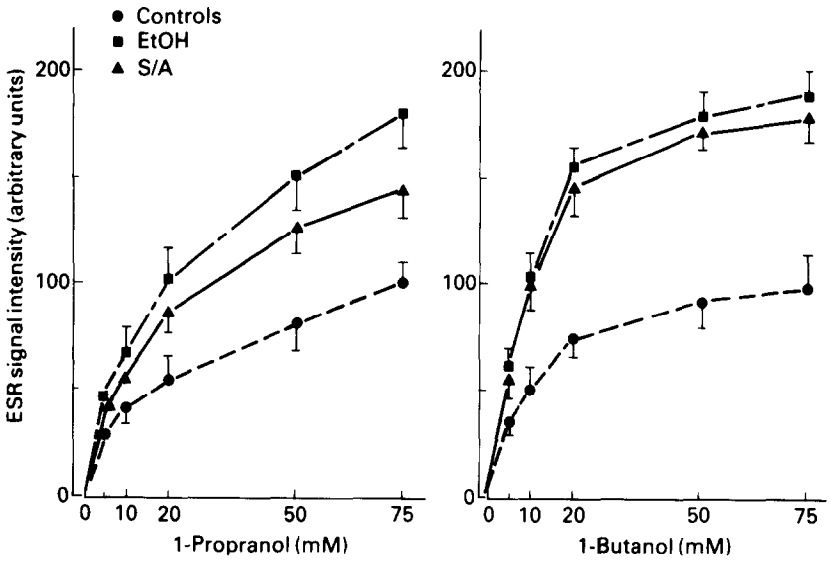

Fig. 2. Concentration-dependent increase in the formation of free radical species from 1-propanol and 1-butanol by liver microsomes from untreated (●), ethanol-fed (■) or starved and acetone pretreated (▲) rats. The results are means of three different experiments ± SD.

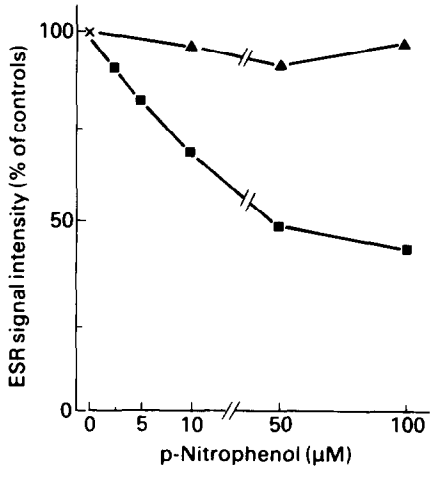

Fig. 3. Effect of increasing concentration of *p*-nitrophenol on the production of 1-hydroxybutyl free radicals by ethanol- (■) or phenobarbital-treated (▲) rat liver microsomes. The results are expressed as per cent of the ESR signal height in the corresponding controls without *p*-nitrophenol.

microsomal fractions of untreated rats and the unilamellar phospholipid vesicles were prepared by cholate gel filtration technique [16] in 15 mM K-phosphate buffer, pH 7.4, plus 50 mM KCl. The vesicles consisted of microsomal phospholipids, NADPH cytochrome P450 reductase and cytochrome P450 in a molar ratio of 1200:0.4:1. After preparation, 0.2 mL of the vesicle suspension, containing about 0.3–0.4 nmoles of cytochrome P450, was immediately incubated for 20 min at 37° with 2–20 mM 1-butanol, 20 mM 4-POBN, 1 mg NADPH in 1 mL (final volume) 50 mM K-phosphate buffer containing 0.1 mM desferrioxamine. The reaction was terminated by the addition of 0.5 mL of the chloroform–methanol mixture and the samples were then processed as described for microsomal preparations.

RESULTS

Evidence for the involvement of cytochrome P450 *in the formation of free radical species from aliphatic alcohols*

Incubation of rat liver microsomes with either 1-propanol or 1-butanol in a medium containing NADPH, the spin trapping agent 4-pyridyl-1-oxide-*t*-butyl nitrone (4-POBN) and 25 μM desferrioxamine resulted in the formation of characteristic ESR spectra due to the trapping of carbon centered free radicals (Table 1). The hyperfine splitting constants of these spectra were essentially identical to those of the free radical adducts produced by the reaction of 20 mM 1-propanol or 1-butanol with hydroxyl radicals generated by the xanthine–xanthine oxidase system (Table 1). These similarities allowed a tentative identification of the spin adducts as due to the trapping of 1-hydroxypropyl and 1-hydroxybutyl free radicals, respectively. The formation of these radical species was strictly dependent upon the presence of NADPH and oxygen and was not influenced by the addition of 25 mM mannitol, a well known scavenger of OH· radicals (not shown).

Following the induction of cytochrome P450IIE1, by the combination of fasting and acetone administration or by chronic alcohol feeding, the production of alcohol-derived radicals by liver microsomes was stimulated about two-fold, as compared to untreated controls (Fig. 1). By contrast, microsomes from phenobarbital-pretreated rats

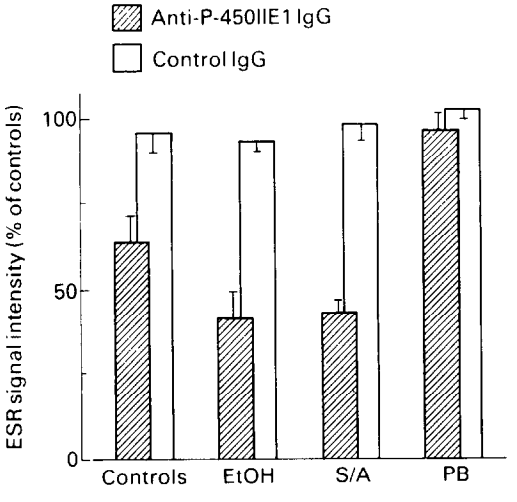

Fig. 4. Inhibition of 1-hydroxybutyl free radical generation in liver microsomes from control, ethanol-fed (EtOH), starvation/acetone (S/A) or phenobarbital (PB) pretreated rats by antibodies raised against cytochrome P450IIE1. Microsomal fractions were preincubated 15 min at 37° with 2 mg/mg protein of either rabbit IgG against cytochrome P450IIE1 or preimmune gammaglobulins. After reaction, the microsomes were separated by centrifugation from the unreacted IgG in order to avoid aspecific reactions with the radicals and subsequently incubated 5 min at 37° in the presence of NADPH, 1-butanol and 4-POBN as reported in the method section. The results are expressed as per cent of the ESR signal intensities in the respective controls incubated without antibodies and are mean of three different experiments ± SD.

produced the same free radical species at rates not higher than control microsomes, despite the increase in the content of cytochrome P450 (Fig. 1). From the same experiments it was clear that the susceptibility of ethanol, 1-propanol and 1-butanol to undergo free radical activation increased with the change in the carbon chain length. This was particularly evident in both ethanol- and acetone-induced microsomes (Fig. 1). These changes are in agreement with the difference in the octanol–water partition coefficients among the three alcohols, probably reflecting a more efficient binding to the hydrophobic region of cytochrome P450. Consistently, titration experiments showed that the growth in the intensity of the ESR signals produced by 1-butanol was apparently saturated at 20 mM concentration, both in control and acetone or ethanol microsomes (Fig. 2). However, no sign of saturation was evident in the case of 1-propanol radical formation (Fig. 2). From these data it emerges that cytochrome P450IIE1 is particularly active in catalysing the activation of aliphatic alcohols to free radical intermediates and that 1-butanol is a good substrate to investigate such a process.

Characterization of the role of cytochrome P450IIE1 *in the free radical metabolism of* 1-*butanol*

The ability of cytochrome P450IIE1 to generate 1-hydroxybutyl radicals was first investigated using *p*-nitrophenol, a specific substrate for this isoenzyme. As shown in Fig. 3 the addition of *p*-nitrophenol in concentrations ranging from 2 up to 50 μM decreased progressively the intensities of the ESR signals produced by ethanol-induced microsomes incubated with 20 mM 1-butanol. By contrast, *p*-nitrophenol was without effect in microsomal preparations from phenobarbital-pretreated rats.

This finding was further supported by experiments performed using antibodies raised against cytochrome P450IIE1. Previous studies have, in fact, demonstrated that these antibodies selectively inhibit the oxidation of ethanol to acetaldehyde by either liver microsomes or reconstituted membrane systems containing this isoenzyme [12, 14]. To investigate the effect of rabbit anti P450IIE1 IgG on the formation of butanol free radicals, microsomal fractions from either untreated, ethanol-, acetone- or phenobarbital-treated rats were preincubated for 15 min with the antibodies or preimmune IgGs (2 mg/mg microsomal protein). After reaction, the microsomes were separated by centrifugation from unbound IgGs in order to avoid aspecific reactions with the radicals and subsequently incubated in the presence of NADPH, 1-butanol and 4-POBN. Figure 4 demonstrates that the preincubation with anti-cytochrome P450IIE1 IgGs decreased the spin trapping of butanol radicals formed by either ethanol- or acetone-induced microsomes by 50%. Under similar conditions, the free radical production by microsomes from untreated animals was diminished by about 35–40%, whereas no effect was seen using microsomes from phenobarbital-treated rats (Fig. 4). In addition no changes in the ESR signals were observed with preimmune IgGs in the different microsomal preparations (Fig. 4). This suggested that the inhibitory effect was due to a specific interaction of IgG with cytochrome P450IIE1.

Role of NADPH oxidase activity of cytochrome P450IIE1 *on the formation of* 1-*butanol free radicals*

Cytochrome P450IIE1 is known to exhibit a high rate of NADPH oxidation and this is reflected in the high amounts of superoxide anion and hydrogen peroxide produced by microsomes prepared from rats exposed to ethanol or acetone [13, 17, 18]. Ekström and Ingelman-Sundberg [19] have recently reported that the generation of reactive oxygen species can be inhibited by 50% using antibodies directed against cytochrome P450IIE1. This effect corresponds to the decrease here observed in the formation of butanol free radicals using the same antibodies and suggests the possibility that the two processes might be related. In a subsequent series of experiments we investigated whether the radical activation of 1-butanol might be influenced by several compounds that have been shown to interfere with the NADPH oxidase activity of cytochrome P450IIE1 [20].

Following the addition of tryptamine, isoniazid and octylamine to ethanol microsomes we observed a dose-dependent decrease in the formation of 1-butanol radicals (Fig. 5). Octylamine was the most active inhibitor and at the concentration of 0.2 mM lowered by about 75% the intensity of the ESR signals. Higher concentrations (2 mM) of tryptamine and isoniazid were instead needed to decrease the

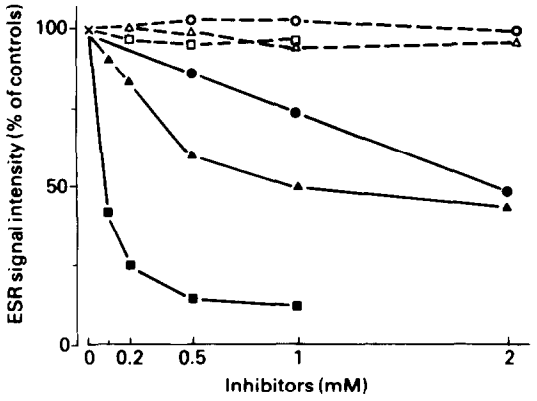

Fig. 5. Effect of cytochrome P450 NADPH oxidase inhibitors on the formation of 1-hydroxybutyl free radical by ethanol-induced rat liver microsomes (filled symbols) or the xanthine–xanthine oxidase system (open symbols). Liver microsomes and the xanthine–xanthine oxidase were incubated as reported in Materials and Methods in the presence of increasing concentration of isoniazid (▲ △), tryptamine (● ○) and octylamine (■ □). The inhibitors were tested in xanthine–xanthine oxidase system to evaluate the possible radical scavenging effect of these compounds. Pargyline 10 μM was added to microsomal incubation containing octylamine in order to inhibit monoamino oxidase activity. The results are expressed as per cent of the ESR signal intensities in the respective controls incubated without inhibitors and are mean of two different experiments in duplicate.

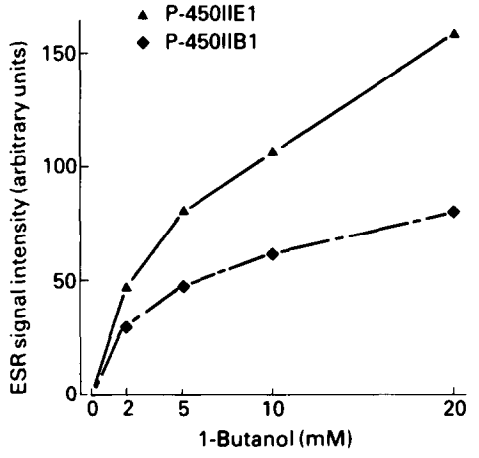

Fig. 6. Free radical activation of 1-butanol in reconstituted membrane vesicles containing cytochrome P450 reductase and, respectively, cytochrome P450IIB1 (◆) or IIE1 (▲). Membrane vesicles containing 0.3–0.4 nmol cytochrome P450 were incubated 20 min at 37° with increasing concentration of 1-butanol and 1 mg NADPH, 20 mM 4-POBN in 50 mM K-phosphate buffer pH 7.4.

signal eights by 50% (Fig. 5). All these compounds were, however, equally unable to affect the formation 1-hydroxybutyl free radicals generated by the xanthine–xanthine oxidase system (Fig. 5). This indicates that their effect was not due to an aspecific scavenging of the alcohol radicals or of any reactive oxygen species.

Free radical activation of butanol as investigated in reconstituted cytochrome P450 *system*

Reconstituted membranes containing NADPH cytochrome P450 reductase and cytochrome P450 have been extensively used for investigating the metabolism of alcohols by the microsomal monoxygenase enzymes. Thus, we employed reconstituted membrane vesicles containing either P450IIE1 or the phenobarbital-inducible form (P450IIB1) to further characterize the mechanism of radical activation of alcohols. When incubated with 1-butanol in the presence of NADPH and 4-POBN, reconstituted systems were found to generate ESR signals identical to those ascribed to the hydroxybutyl free radical (Table 1). The free radical formation was strictly dependent upon the presence of cytochrome P450 and no detectable ESR spectra were observed when vesicles containing NADPH cytochrome P450 reductase alone were incubated with NADPH, 1-butanol and 4-POBN (not shown).

As observed in liver microsomes, the intensity of the ESR signals produced by reconstituted systems increased with the concentration of 1-butanol added and cytochrome P450IIE1 was about twice as active as P450IIB1 in catalysing the free radical activation process (Fig. 6).

The possible involvement of the oxidase activity of cytochrome P450 in the generation of alcohol radicals was investigated in the reconstituted membrane systems containing both P450IIE1 and P450IIB1 and using octylamine (0.1 mM), isoniazid (1 mM) or tryptamine (1 mM) as inhibitors. From Fig. 7 it appears that, in the presence of isoenzyme IIE1, these compounds were able to decrease the formation of butanol radicals by 50–60%, whereas only octylamine was capable to exert an appreciable effect in the vesicles containing cytochrome P450IIB1 (Fig. 7). Moreover, the decrease in the ESR signal intensities produced by isoniazid, tryptamine or octylamine corresponded to the inhibition of cytochrome P450IIE1-dependent oxidase activity previously observed by Persson and co-workers [20].

DISCUSSION

In recent years, the combined use of ESR spectroscopy and spin trapping technique has demonstrated the production of free radical intermediates during the microsomal oxidation of aliphatic alcohols [7, 8]. It has been possible to distinguish two pathways leading to the formation of hydroxyethyl radical from ethanol [8, 21]. One pathway requires the presence of traces of non haeme iron and is consistent with a hydroxyl radical-mediated oxidation of alcohols as previously described [3–8, 17, 21], whereas the other is apparently independent from the presence of hydroxyl radicals [8, 22]. The decrease of the hydroxyethyl radical formation by cytochrome P450 blockers has suggested the involvement of this haemoprotein in the $OH^{\cdot}$-independent activation process [8]. This is supported by the present findings

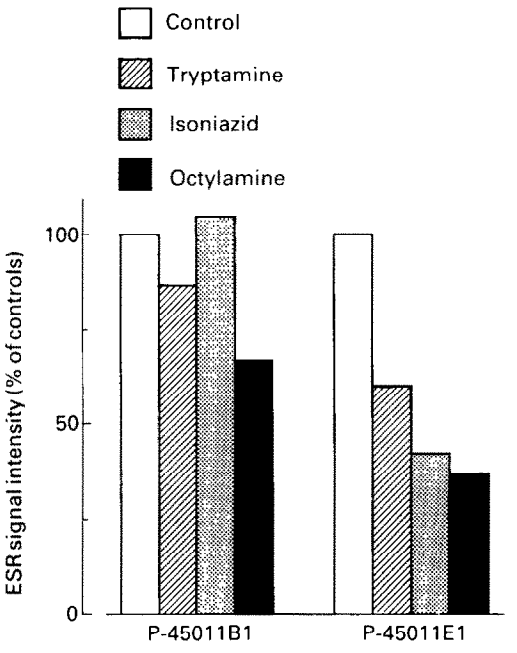

Fig. 7. Inhibition of 1-butanol free radical activation in reconstituted membrane vesicles containing cytochrome P450 reductase and, respectively, cytochrome P450IIB1 or P450IIE1 by inhibitors of cytochrome P450 NADPH oxidase activity. Reconstituted vesicles were incubated 20 min at 37° with 10 mM 1-butanol, 1 mg NADPH, 20 mM 4-POBN and, respectively, 1 mM isoniazid and tryptamine or 0.1 mM octylamine in 50 mM K-phosphate buffer pH 7.4 containing 0.1 mM desferrioxamine. The results are expressed as per cent of the ESR signal intensities in the respective controls not receiving the inhibitors and are mean of two experiments in duplicate.

that reconstituted membrane vesicles only containing cytochrome P450 reductase were unable to produce 1-hydroxybutyl radicals when incubated with NADPH in the presence of 1-butanol and 4-POBN. Moreover, the capability of different alcohols to form free radical increases with the length of the carbon chain, suggesting that the free radical formation is taking place in an hydrophobic environment, most likely in the neighbourhood of the cytochrome P450 active side. Such an interpretation is consistent with previous results by Morgan and colleagues [10] showing that the rates of alcohol oxidation by ethanol-inducible cytochrome P450 correlate with the water–octanol partition coefficients of the various alcohols. However, a direct comparison between these variables might be difficult to obtain, since the availability of the spin trap to the active site of cytochrome P450 must be different from that of alcohols.

The preferential role of the ethanol-inducible cytochrome P450IIE1, to mediate the oxidation of ethanol as well as of other aliphatic alcohols has been documented in a number of studies. For instance, oxidations of ethanol, 1-propanol and 1-butanol in reconstituted systems were 2–4 times higher with cytochrome P450IIE1 in comparison to other isoenzymes [10]. Immunochemical studies have revealed that the major part of the ethanol metabolism by liver microsomes from either ethanol-treated rabbits or human alcoholic patients is due to the action of this form of cytochrome P450 [10, 12, 17]. Consistent with these observations, we have found that the formation of free radical intermediates from alcohols is stimulated by the induction of cytochrome P450IIE1 following either chronic ethanol feeding of the rats or by the combination of fasting and acetone pretreatment (Fig. 2). Furthermore, a selective inhibition of 1-butanol radicals generation is produced by *p*-nitrophenol, a substrate of this isoenzyme, as well as by antibodies directed against P450IIE1. In most of the cases the ESR signals were not decreased by more than 50%, suggesting that some other forms of cytochrome P450 might be responsible for the remaining fraction of the free radical generation. However, it is evident that the capacity of these antibodies to inhibit microsomal ethanol oxidation [14] is similar as here seen for the effect on radical generation. By contrast, the effectiveness of the same antibodies in inhibiting other cytochrome P450IIE1 reactions is somewhat greater with substrates like benzene and carbon tetrachloride [14].

Free radical intermediates are known to be produced during cytochrome P450-catalysed activation of several classes of xenobiotic, but none of the mechanisms postulated for such reactions seem to apply to the formation of alcohol-derived free radicals. It has been proposed that hypervalent ferryl cytochrome P450 complex (P450–$[FeO]^{3+}$) catalyses most monoxygenase reactions by sequential one-electron steps with the formation of free radical intermediates [24]. The evidence so far available, however, do not support the possibility that these intermediates are released by the cytochrome and conversely suggest that the radical species further reacts at the site of formation to give a non-radical product [24].

Using both liver microsomes or reconstituted membrane systems containing P450IIE1 we have observed that the addition of tryptamine, isoniazid and octylamine, effectively inhibits the production of 1-butanol radical. Interestingly, the above compounds are the only active among 30 different chemicals previously tested for their ability to inhibit cytochrome P450IIE1 dependent oxidase activity measured as H_2O_2 formation and NADPH oxidation [20]. The isoenzyme selectivity of the above compounds was complete since none of them affected cytochrome P450IIB4 oxidase activity [20].

During the oxidase activity of the microsomal monoxygenase system hydrogen peroxide originates from the dismutation of superoxide anion formed by the auto-oxidation of the oxycytochrome complex [25, 26]. In the presence of iron these oxygen metabolites can undergo the so called iron-catalysed Haber-Weiss reaction leading to the formation of highly reactive hydroxyl radical ($OH^{\cdot}$) which can contribute to the microsomal oxidation of several compounds including ethanol [3–5, 17], benzene [27] and aniline [28].

In the conditions of the experiments here described the participation of iron to the formation of $OH^{\cdot}$

should be minimal because of the presence of the iron chelating agent desferrioxamine [8]. Moreover, the increase in the amounts of alcohol radicals spin trapped with the change in the carbon chain length is incompatible with a process mediated by free hydroxyl radicals because the reaction rates of $OH^{\cdot}$ with different alcohols are quite similar [29]. Therefore, the formation of alcohol free radicals should be due to an oxidizing species possibly bound to the cytochrome P450 and sufficiently reactive to abstract a proton from the alcohol alpha-carbon. Tryptamine, isoniazid and octylamine, which interact with the cytochrome and induce a shift of haeme iron from high to low spin, thereby making the formation of oxycytochrome P450 complex impossible, inhibit to the same extent the formation of both the 1-hydroxybutyl radical and superoxide anion by cytochrome P450IIE1. This indicates that the generation of alcohol radicals should occur during the process of univalent oxygen reduction. Thus, we suggest the possibility that the ferric cytochrome P450–oxygen complex (cyt-P450–Fe^{3+} O_2^-) formed during the reduction of haeme–oxygen might react with the alcohol molecule and subsequently release a carbon centered radical and H_2O_2. The reactivity of cyt-P450–$Fe^{3+}O_2^-$ should then be comparable to that of the protonated form of superoxide, perhydroxyl radical (HO_2), rather than that of superoxide itself. This hypothesis is also compatible with the observation that cytochrome P450IIE1 which exhibits a high rate of oxidase activity [18], is more active than the isoenzyme IIB1 in forming alcohol free radicals.

In conclusion, the results presented demonstrate that cytochrome P450IIE1 is specially active in forming alcohol free radical intermediates and suggest the possibility that such activation pathway might be related to the capacity of this isoenzyme to reduce dioxygen. If this is the case the production of free radical species might occur as a side reaction in the process of alcohol oxidation to aldehydes by the microsome mono-oxygenase system. The production of hydroxyethyl radicals from ethanol could have a particular pathophysiological relevance in explaining the oxidative lesions often observed following alcohol intoxication [30], specially in light of the recent observation that these radical species can be spin trapped *in vivo* in the bile of ethanol treated deer-mice [22].

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REFERENCES

1. Orme-Johnsson WH and Ziegler DM, Alcohol mixed function oxidase activity of mammalian liver microsomes. *Biochem Biophys Res Commun* **21**: 78–82, 1965.
2. Ohnishi K and Lieber CS, Reconstitution of the microsomal-oxidizing system: qualitative and quantitative changes of cytochrome P450 after chronic ethanol consumption. *J Biol Chem* **252**: 7124–4131, 1977.
3. Cederbaum AI and Dicker E, Inhibition of microsomal oxidation of alcohols by hydroxyl radical scavenging agents by the iron-chelating agent desferrioxamine. *Biochem J* **210**: 107–113, 1983.
4. Winston GW and Cederbaum AI, NADPH-dependent production of oxy radicals by purified components of rat liver mixed function oxidase system II. Role in microsomal oxidation of ethanol. *J Biol Chem* **258**: 1514–1519, 1983.
5. Cederbaum AI, Qureshi A and Messenger P, Oxidation of isopropanol by rat liver microsomes: possible role of hydroxyl radicals. *Biochem Pharmacol* **30**: 825–831, 1981.
6. Krikun G and Cederbaum AI, Stereochemical studies on the cytochrome P450 and hydroxyl radical dependent pathways of 2-butanol oxidation by microsomes from chow-fed, phenobarbital-treated and ethanol treated rats. *Biochemistry* **23**: 5489–5494, 1984.
7. Albano E, Tomasi A, Goria-Gatti L, Poli G, Vannini V and Dianzani MU, Free radical metabolism of alcohols by rat liver microsomes. *Free Rad Res Commun* **3**: 243–249, 1987.
8. Albano E, Tomasi A, Goria-Gatti L and Dianzani MU, Spin trapping of free radical species produced during the microsomal metabolism of ethanol. *Chem Biol Interact* **65**: 223–234, 1988.
9. Ekström G, Norsten C, Cronholm T and Ingelman-Sundberg M, Cytochrome P450 dependent ethanol oxidation. Kinetic isotope effects and absence of stereoselectivity. *Biochemistry* **26**: 7348–7354.
10. Morgan ET, Koop DR and Coon MJ, Catalytic activity of cytochrome P450 isoenzyme 3a isolated from liver microsomes of ethanol-treated rabbits. *J Biol Chem* **257**: 13951–13957, 1982.
11. Koop D, Nordblom GD and Coon MJ, Immunochemical evidence for a role of cytochrome P450 in liver ethanol oxidation. *Arch Biochem Biophys* **235**: 228–238, 1984.
12. Ekström G, Von Bahr C and Ingelman-Sundberg M, Human liver microsomal cytochrome P450IIE1. Immunological evaluation of its contribution to microsomal ethanol oxidation, carbon tetrachloride reduction and NADPH oxidase activity. *Biochem Pharmacol* **38**: 689–693, 1989.
13. Ekström G, Cronholm T and Ingelman-Sundberg M, Hydroxyl-radical production and ethanol oxidation by liver microsomes isolated from ethanol-treated rats. *Biochem J* **233**: 755–761, 1986.
14. Johansson I, Ekström G, Scholte B, Puzychi D, Jörnvall H and Ingelman-Sundberg M, Ethanol-, fasting, and acetone-inducible cytochromes P450 in rat liver: regulation and characteristics of enzyme belonging to the IIB and IIE gene subfamilies. *Biochemistry* **28**: 1925–1934, 1988.
15. De Carli LM and Lieber CS, Fatty liver in the rat after prolonged intake of ethanol with nutritionally adequate new liquid diet. *J Nutr* **91**: 331–336, 1967.
16. Ingelman-Sundberg M and Glaumann H, Incorporation of purified components of rabbit liver microsomal hydroxylase system into phospholipid vesicles. *Biochim Biophys Acta* **599**: 417–431, 1980.
17. Ingelman-Sundberg M and Johansson I, Mechanisms of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochrome P450. *J Biol Chem* **259**: 6447–6458, 1984.
18. Gorsky LD, Koop DR and Coon MJ, On the stoichiometry of the oxidase and monoxygenase reactions catalysed by liver microsomal cytochrome P450. *J Biol Chem* **259**: 6812–6817, 1984.
19. Ekström G and Ingelman-Sundberg M, Rat liver microsomal NADPH-supported oxidase activity and

lipid peroxidation dependent on ethanol-inducible cytochrome P450 (P459IIE1). *Biochem Pharmacol* **38**: 1313–1318, 1989.
20. Persson JO, Terelius Y and Ingelman-Sundberg M, Cytochrome P450-dependent formation of oxygen radicals. Isoenzyme-specific inhibition of P450-mediated reduction of oxygen and carbon tetrachloride. *Xenobiotica* **20**: 887–900, 1990.
21. Reinke LA, Rau JM and McCay PB, Possible roles of free radicals in alcoholic tissue damage. *Free Rad Res Commun* **9**: 205–211, 1990.
22. Knecht KT, Bradfort BU, Mason RP and Thurman RG, *In vivo* formation of free radical metabolite of ethanol. *Mol Pharmacol* **38**: 26–30, 1990.
23. Ortiz de Montellano PR, Cytochrome P450 catalysis: radical intermediates and dehydrogenation reactions. *TIBS* **10**: 354–359, 1989.
24. Albano E, Cheeseman KH, Tomasi A, Carini R, Dianzani MU and Slater TF, Effect of spin traps in isolated rat hepatocytes and liver microsomes. *Biochem Pharmacol* **35**: 3955–3960, 1986.
25. Kutham H, Tsuji H, Graf H, Ullrich V, Werringloer J and Estabrook RW, Generation of O_2^- in a reconstituted mono-oxygenase system. *FEBS Lett* **91**: 343–345, 1978.
26. Terelius Y and Ingelman-Sundberg M, Cytochrome P450-dependent oxidase activity and hydroxyl radical production in micellar and membranous types of reconstituted systems. *Biochem Pharmacol* **37**: 1383–1389, 1988.
27. Ingelman-Sundberg M and Hagbjörk AL, On the significance of the cytochrome P450-dependent hydroxyl radical mediated oxygenation mechanism. *Xenobiotica* **12**: 673–686, 1982.
28. Ingelman-Sundberg M and Ekström G, Aniline is hydroxilated by a cytochrome P450 dependent hydroxyl radical mediated oxygenation mechanism. *Biochem Biophys Res Commun* **106**: 625–631, 1982.
29. Willson RL, Free radicals and tissue damage: mechanistic evidence from radication studies. In: *Biochemical Mechanisms of Liver Injury* (Ed. Slater TF), pp. 124–224. Academic Press, London, 1979.
30. Albano E, Poli G, Tomasi A and Dianzani MU, Free radical mechanisms in alcohol-mediated liver injury. In: *Chronic Liver Damage* (Eds. Dianzani MU and Gentilini P), pp. 27–38. Excerpta Medica, Amsterdam, 1990.