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POSSIBLE ROLES OF FREE RADICALS IN ALCOHOLIC TISSUE DAMAGE

LESTER A. REINKE*

Department of Pharmacology, University of Oklahoma Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190

JAMES M. RAU and PAUL B. McCAY

Molecular Toxicology Research Group, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104

Hepatic microsomes metabolize ethanol to a free radical metabolite which forms adducts with the spin trapping agents PBN (phenyl-N-t-butylnitrone) and DMPO (5,5-dimethyl-l-pyrroline N-oxide). This ethanol radical has been identified as the l-hydroxyethyl radical through the use of ^{13}C-labelled ethanol. A role of the cytochrome P-450 enzymes in the generation of the l-hydroxyethyl radical was suggested by requirements for oxygen and NADPH, as well as inhibition in the presence of SKF 525-A and imidazole.

In contrast, the ESR signal intensity of the l-hydroxyethyl radical was diminished when either catalase, or the iron chelating agent deferoxamine, was added to the microsomal incubations, and was increased by the addition of ADP-Fe. These observations suggest that the ethanol radicals may arise secondary to iron-catalyzed formation of hydroxyl radicals from hydrogen peroxide. This possibility was supported by enhanced rates of l-hydroxyethyl radical formation when microsomal catalase activity was inhibited by the addition of sodium azide, or by pretreatment of rats with aminotriazole. However, the reaction was relatively insensitive to scavengers of the hydroxyl radical. Thus, the mechanism of l-hydroxyethyl radical formation could involve two cytochrome P-450-dependent pathways: generation of hydrogen peroxide required for a Fenton reaction, as well as direct catalytic formation of the ethanol radical.

KEY WORDS: l-Hydroxyethyl radical, spin trapping, ethanol administration, microsomal oxidation, hydroxyl radical, cytochrome P-450.

INTRODUCTION

Chronic abuse of alcoholic beverages leads to pathological changes in a number of tissues. The liver is especially sensitive to the deleterious effects of ethanol, but the mechanism(s) for development of alcoholic liver disease remain unclear. Many investigators have proposed a role of lipid peroxidation in this phenomenon, based on increased levels of malondialdehyde, conjugated dienes, and chemiluminescence after the acute and chronic administration of ethanol to laboratory animals.[1,2]

Because lipid peroxidation often occurs as a result of free radical attack on membrane lipids, potential sources of initiating radicals have been explored. Several investigators have suggested that liver microsomes isolated from ethanol-fed rats produce higher concentrations of superoxide anion, hydrogen peroxide, and hydroxyl radicals than microsomes from untreated rats.[3-5] Liver microsomes are also known to metabolize ethanol to the l-hydroxyethyl radical.[6,7] If these free radical species are

*Author for correspondence.

also produced in the endoplasmic reticulum *in vivo*, lipid peroxidation would be a likely consequence.

We have recently reported that carbon-centered radicals can be detected in livers of rats fed ethanol-containing liquid diets for two weeks.[6] In these experiments, the spin trapping agent 2,4,6-trimethoxyphenyl-N-t-butylnitrone was administered to rats, and electron spin resonance (ESR) spectra which are thought to be due to lipid radical adducts of the spin trap were observed in extracts of livers from ethanol-fed rats, but in samples from pair-fed controls. The intensity of radical formation was increased when rats were fed diets high in fat[6] which is significant because dietary fat is known to increase the severity of alcoholic liver damage.

The 1-hydroxyethyl radical must be considered as a potentially toxic metabolite of ethanol. However, little is known of the mechanism for the bioactivation of ethanol to a free radical. The current study has been designed to explore factors affecting rates of 1-hydroxyethyl radical formation in hepatic microsomes.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing 140 to 150 grams were purchased from Sasco (Omaha, NE), and were fed lab chow *ad libitum* until used in experiments. Livers were homogenized in 0.15 M potassium phosphate buffer, pH 7.4, microsomes were isolated by differential centrifugation, and were washed twice by resuspension in the phosphate buffer. The microsomes (1 to 2 mg protein/ml) were incubated at 37°C with 50 mM ethanol, 30 mM PBN, and an NADPH-generating system as previously described.[6] In some experiments, DMPO (130 mM) was substituted for PBN. After 15 to 30 minutes of incubation, the reaction systems were extracted with toluene or benzene.

ESR spectra were recorded at room temperature with either an IBM-Bruker ESP 300 or a Varian E-9 EPR spectrometer equipped with an X-band microwave bridge. Instrumental conditions are given in the figure legends.

Catalase activity in liver fractions was measured by recording the rate of decrease of hydrogen peroxide absorbance at 240 nm, following the addition of tissue samples. Hydrogen peroxide generation by microsomes was measured by the ferrithiocyanate method utilized by Kuthan and Ullrich.[8]

RESULTS

When ethanol was incubated with rat liver microsomes, ethanol, PBN, and an NADPH-generating systems, the ESR spectrum of the 1-hydroxyethyl radical adduct of PBN could be observed in toluene extracts (Figure 1). When sodium azide, an inhibitor of catalase, was added to the incubation mixture, the intensity of the ESR signal was increased nearly two-fold (Figure 1). Because the signal intensity is proportional to the concentration of free radical adducts in the sample, these results indicate that azide has stimulated rates of formation of the 1-hydroxyethyl radical.

In the absence of azide, very little hydrogen peroxide could be detected in microsomal incubations with NADPH under aerobic conditions. However, addition of sodium azide (1 mM) resulted in an average hydrogen peroxide generation rate of 1.9 nmoles/min/mg of microsomal protein.

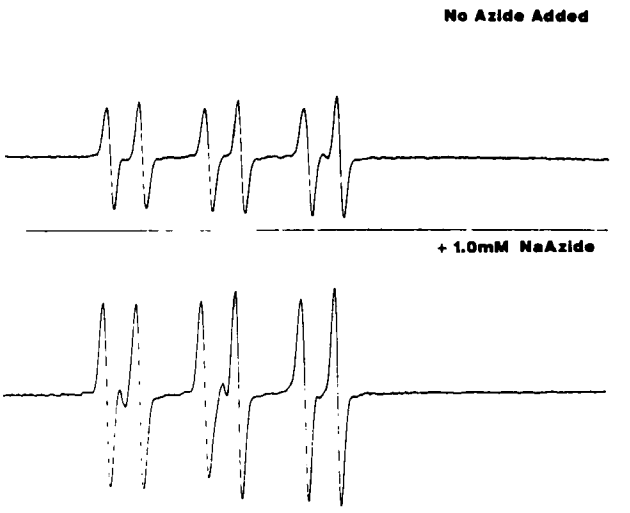

FIGURE 1 Effect of Sodium Azide on the Formation of the 1-Hydroxyethyl Radical Adduct of PBN During the Metabolism of Ethanol by Rat Liver Microsomes. Ethanol was incubated with microsomes, PBN, and an NADPH-generating system as indicated in Materials and Methods, and the system was extracted with toluene after 20 minutes of incubation. In the lower panel, sodium azide was included at a final concentration of 1 mM. The IBM-Bruker ESP 300 spectrometer settings for both spectra were as follows: microwave power, 19.8 mW; modulation amplitude, 0.975 G; gain, 2×10^4; time constant, 0.33 sec; scan range, 100 G; and scan time. 335 sec. For these spectra, $a_N = 15.1$ G; $a_\beta^H = 5.0$ G.

Results with azide indicated that hydrogen peroxide could be involved in the microsomal generation of the 1-hydroxyethyl radical. This hypothesis was tested by treating rats with 3-amino-1,2,4-triazole (AT), which is metabolized *in vivo* to an irreversible inhibitor of catalase.[9] The AT was administered at a dose of 1 g/kg, i.p., 60 minutes prior to removal of livers and preparation of microsomes. This dose of AT has been shown to abolish the spectral changes of catalase compound I in perfused rat livers.[10] Under these conditions, AT treatment inhibited catalase activity in liver homogenates by > 90%. The ESR spectra of the 1-hydroxyethyl radical adducts of PBN were 53% more intense in microsomes from AT-treated rats than in microsomes of rats which had received an equivalent volume of saline (data not shown). However, addition of azide did not further increase the intensity of signals in microsomes from AT-treated rats. These data demonstrate that the stimulatory effect of azide depicted in Figure 1 is the result of inhibition of catalase contaminating the microsomes.

Mechanisms of 1-hydroxyethyl radical generation by microsomes have been probed by altering conditions of the incubation system, and measuring the effects on the signal intensity (see Figure 1). No signals could be detected if the incubations were performed under an atmosphere of argon, if NADPH was omitted from the incubation, or if the microsomes were placed in a boiling water bath for three minutes prior to incubation. The cytochrome P-450 inhibitors SKF 525-A and imidazole both decreased the intensity of the ESR signals (Table 1). Addition of catalase, deferoxamine, reduced glutathione, and excess hydrogen peroxide decreased the intensity of the 1-hydroxyethyl radical adduct signal, while adition of ADP-Fe increased the signal intensity. The addition of mannitol or dimethyl sulfoxide had little effect on ethanol radical formation (Table 1).

TABLE I

Effects of various additions on the formation of 1-hydroxyethyl radical adducts of PBN during microsomal ethanol metabolism

Addition	Relative ESR signal
None	100
SKF 525-A (1 mM)	48
Imidazole (1 mM)	67
Catalase (100 units/ml)	42
Hydrogen Peroxide (1 mM)	68
ADP-Fe^{3+} (0.4 mM–0.012 mM)	201
Deferoxamine (1.0 mM)	32
Mannitol (10 mM)	88
Dimethyl Sulfoxide (10 mM)	95
Reduced Glutathione (10 mM)	25

Ethanol was incubated with microsomes, PBN, and an NADPH-generating system, as indicated in Materials and Methods. On each day of experiments, the signal intensity of the 1-hydroxyethyl radical (see Figure 1) in the absence of other additions was assigned a value of 100, and the value for the relative ESR signal was assigned from the ratio of the peak signal heights. The values are means of at least two experiments.

In some experiments, DMPO was substituted for PBN in order to test for the presence of oxygen-centered radicals during the generation of the 1-hydroxyethyl radical. With DMPO as a spin trap, a spectrum with six spectral peaks was observed in benzene extracts of microsomes metabolizing ethanol (Figure 2). If 1-^{13}C-ethanol was used in the experiments, splitting of the spectral peaks occurred, indicating that the adduct trapped was the 1-hydroxyethyl radical. When ethanol was omitted from the incubation system, no ESR signal was observed (not shown).

DISCUSSION

The microsomal metabolism of ethanol to acetaldehyde was first reported by Orme-Johnson and Ziegler.[11] Since that time, the mechanisms of this oxidation have been the subject of intense debate. The role of cytochrome P-450 in ethanol oxidation has been proven with experiments utilizing purified cytochrome P-450 and reductase.[12] However, there is considerable evidence which suggests that hydroxyl radicals could have a role in microsomal ethanol metabolism. For example, the oxidation of dimethyl sulfoxide and 2-keto-4-thiomethylbutyric acid can be catalyzed by hydroxyl radicals, and cross competition in the microsomal oxidation of these substrates and ethanol has been reported.[5] Hydroxyl radicals are known to react rapidly with ethanol to abstract a hdyrogen atom, forming water and the 1-hydroxylethyl radical.

Although generation of 1-hydroxyethyl radicals from ethanol by liver microsomes have been proven with the spin trapping agents PBN (Figure 1), DMPO (Figure 2), and 4-pyridyl-1-oxide-t-butylnitrone,[7] the mechanism for this metabolism remains unclear. The involvement of cytochrome P-450 is indicated by the dependence on NADPH and oxygen, and sensitivity to SKF 525-A and imidazole (Table 1). Albano *et al.*, have also demonstrated an inhibitory effect of metyrapone and 1-nitroisothiocyanate.[7] But other data strongly suggest an important role of hydrogen peroxide in the microsomal formation of ethanol radicals.

It is well established that hydrogen peroxide is formed by hepatic microsomes in the

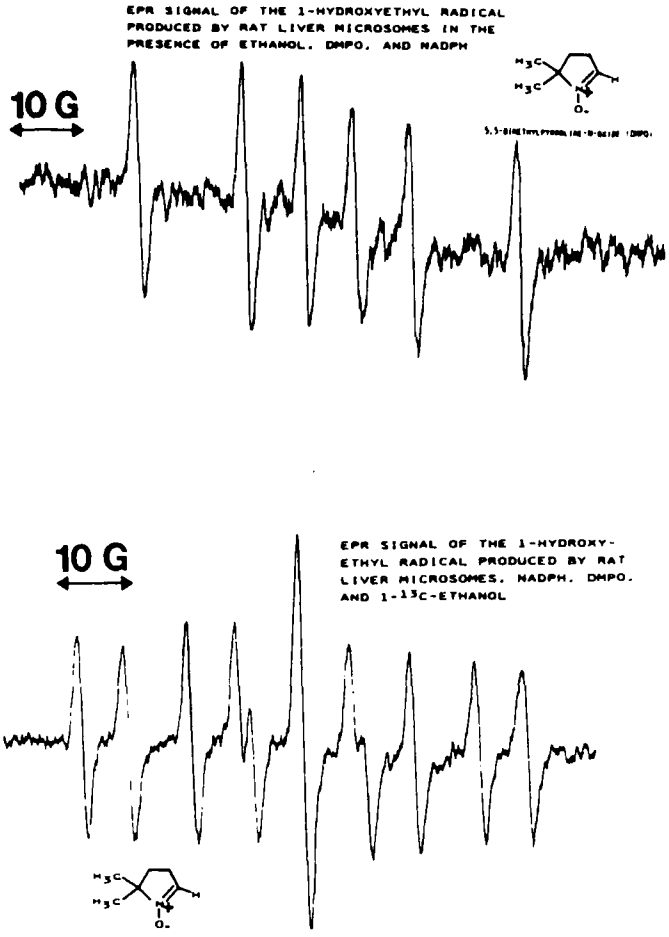

FIGURE 2 Spectra of the 1-Hydroxyethyl Radical Adduct of DMPO Formed by Rat Liver Microsomes. Ethanol was incubated with microsomes, DMPO, and an NADPH-generating system. After 30 minutes of incubation, the systems were extracted into benzene. In the lower panel, 1-^{13}C-ethanol was used as the substrate. The Varian E-9 spectrometer settings were microwave power, 25 mW; time constant, 3 sec; scan range, 100 G; scan time, 16 min; modulation amplitude, 1.0 G; and gain, 5×10^4. For the spectra obtained with ^{13}C-ethanol, the modulation amplitude was 2.0 G and the gain was 4×10^4. For these spectra, $a_N = 14.6$ G; $a_\beta^H = 22.5$ G; and $a_{C\text{-}13} = 6.2$ G.

presence of oxygen and NADPH,[8] and the cytochrome P-450 enzymes have been shown to be its major source. Under normal conditions, the hydrogen peroxide is rapidly catabolized by catalase, which contaminates the microsomal fraction after homogenization of the liver. However, the concentration of hydrogen peroxide can be increased substantially through inhibition of catalase by azide or by pretreatment of rats with aminotriazole. The increases in 1-hydroxyethyl radical formation in the presence of azide (Figure 1), or when microsomes from AT-treated rats were used (Results), as well as the inhibitory effects of excess catalase (Table 1) all indicate an important role of hydrogen peroxide in the formation of ethanol radicals. In addition,

the stimulation of adduct formation by ADP-Fe and inhibitory effects of deferoxamine, a potent and specific iron chelating agent (Table 1), further suggest that hydroxyl radicals formed in the presence of iron and hydrogen peroxide may drive microsomal ethanol radical formation.

These observations raise the question of whether hydrogen peroxide-dependent reactions provide the only pathway for 1-hydroxyethyl radical formation in microsomes. Several lines of evidence suggest that this may not be the case. First, there is substantial residual activity in the presence of excess catalase and deferoxamine (Table 1). Similar results were obtained by Albano *et al.*, who also showed that the stimulatory effects of azide were lost when care was taken to deplete the incubation systems of iron.[7] Secondly, mannitol and dimethyl sulfoxide, which are commonly utilized scavengers of the hydroxyl radical, had relatively small effects on the formation of ethanol radicals (Table 1). Third, the spectra of hydroxyl radical adducts were not observed in microsomes metabolizing ethanol (Figure 2). And finally, addition of excess hydrogen peroxide, which could be expected to generate high concentrations of hydroxyl radicals, inhibited the formation of 1-hydroxyethyl radicals (Table 1). This inhibitory effect of hydrogen peroxide could be explained by oxidative damage to some other enzymatic system which is also involved in ethanol radical formation.

Thus, these data suggest that there are at least two pathways for ethanol metabolism to a free radical in hepatic microsomes. One pathway utilizes hydrogen peroxide and may involve hydroxyl radicals, and a second source may be direct generation by the cytochrome P-450 enzymes. Hydrogen atom abstraction at the active site of cytochrome P-450 has been demonstrated for some substrates.[13]

Another important question is the fate of 1-hydroxyethyl radicals that may be formed *in vivo* during periods of ethanol intoxication. Although 1-hydroxethyl radical adducts of PBN (Figure 1) and DMPO (Figure 2) are readily formed in hepatic microsomes, we have been unable to observe these signals in livers of rats given the spin trapping agents and a large dose of ethanol.[6,14] However, in these experiments, ESR signals thought to be due to lipid radical adducts were observed in extracts of liver, heart, lung and spleen of rats which had been treated with ethanol, but not in tissues of rats which had received an equivalent volume of saline and the spin trap.[6,14] Therefore, it is conceivable that the attack of ethanol radicals on cellular membranes could have resulted in formation of other free radical intermediates in the various organs. It is also obvious that glutathione, at physiological concentrations, dramatically decreases the intensity of the 1-hydroxyethyl radical signal in microsomes (Table 1), as previously observed by Albano *et al.*[7] Thus, spin trapping of the 1-hydroxyethyl radical *in vivo* may be unsuccessful due to the effects of glutathione and perhaps other cellular defense mechanisms.

Acknowledgements

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