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HYDROXYL RADICALS ARE GENERATED BY HEPATIC MICROSOMES DURING NADPH OXIDATION: RELATIONSHIP TO ETHANOL METABOLISM

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Ethanol is metabolized to acetaldehyde by hepatic microsomes in a reaction that requires cytochrome P-450, and a role for hydroxyl radicals has been implicated in this process. However, previous spin trapping experiments have failed to demonstrate the production of hydroxyl radicals by liver microsomes unless iron or other metal catalysts have been added. The spin trapping experiments described in this report provide unambiguous evidence that liver microsomes form hydroxyl radicals during oxidation of NADPH, that the addition of exogenous iron is unnecessary for this process, and that hydroxyl radicals participate in the metabolism of ethanol. Liver microsomes are known to metabolize ethanol to the 1-hydroxyethyl radical, and our experimental data support the conclusion that a significant part of the production of the 1-hydroxethyl radical occurs as a consequence of hydroxyl radical attack on ethanol. Lack of previous observation of microsomal hydroxyl radical production in spin trapping experiments is shown to be related to the contamination of the microsomes with catalase.

KEY WORDS: Hydroxyl radical, 1-hydroxyethyl radical, ethanol, spin trapping, free radicals, hepatic microsomes, catalase, hydrogen peroxide.

INTRODUCTION

The primary metabolic fate of ethanol is oxidation to acetaldehyde by enzymes found in the liver. The predominant pathway for ethanol metabolism is via cytosolic alcohol dehydrogenase activity. Ethanol can also be peroxidatively metabolized to acetaldehyde by catalase when hydrogen peroxide is present,[1] but the contribution of this pathway to ethanol metabolism is questionable because of the low intracellular concentrations of H_2O_2.[2,3] In 1965, Orme-Johnson and Ziegler reported that hepatic microsomes also metabolized ethanol to acetaldehyde.[4] It was first proposed that microsomal ethanol oxidation was catalyzed by the drug-metabolizing enzumes such as cytochrome P-450,[5] but it was also recognized that at least some of the acetaldehyde could be formed peroxidatively by catalase contaminating the microsomes, because microsomal enzymes continually form H_2O_2 in the presence of NADPH.[6] The observation that highly purified cytochrome P-450 enzymes in reconstituted systems were

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capable of oxidizing ethanol to acetaldehyde clearly demonstrated a catalase-independent mechanism for microsomal ethanol metabolism.[7,8]

However, the molecular mechanisms for the cytochrome P-450 dependent metabolism of ethanol remain uncertain, because results from a number of laboratories have indicated that hydroxyl radicals may be involved in this reaction.[9-14] It was proposed that hydrogen peroxide generated by the microsomal enzymes, in the presence of iron chelates which are invariably present in the incubation system, results in hydroxyl radical generation through a Fenton-type reaction. This hypothesis was formulated on the basis of indirect evidence involving hydroxyl radical "scavengers", iron chelation, etc. Hydroxyl radicals readily attack ethanol, abstracting a hydrogen atom to form the 1-hydroxethyl radical, which is thought to undergo subsequent reactions lto ultimately produce acetaldehyde. More recently, definitive evidence for the production of 1-hydroxyethyl radicals during microsomal ethanol oxidation has been obtained using the combined technologies of spin trapping and electron paramagnetic resonance spectroscopy.[15-18] A role for hydrogen peroxide in 1-hydroxyethyl radical formation was suggested by inhibitory effects of added catalase or deferoxamine, and increased rates of ethanol radical formation after inhibition of microsomal catalase activity or the addition of hydrogen peroxide.[16,18]

A difficulty with the hypothesis that microsomal ethanol oxidation is mediated through a hydroxyl radical intermediate is that most spin trapping experiments have failed to demonstrate hydroxyl radical formation. For example, microsomes formed 1-hydroxyethyl radical adducts of DMPO in the presence of ethanol, but no adducts were observed in the absence of ethanol.[18] Similarly, Lai and Piette have utilized spin trapping to study free radical formation during microsomal lipid peroxidation, but observed hydroxyl radical adducts only when iron was added to the microsomal incubations.[19-21] These negative results could result from inadequate methodology for the study of microsomal hydroxyl radical formation, or could indicate that oxidizing intermediates other than hydroxyl radicals are responsible for ethanol radical generation. An understanding of the mechanism through which ethanol is metabolized to the 1-hydroxyethyl radical *in vitro* is essential for the determination of the extent to which generation of these radicals may contribute to the toxicity of ethanol *in vivo*.[22,23]

The results presented in this report demonstrate that hydroxyl radicals are generated by hepatic microsomes oxidizing NADPH, and that previous negative results are most likely explained by rapid catabolism of hydrogen peroxide by catalase which contaminates even thoroughly washed, Sepharose CL-2B chromatographed microsomes. These observations are consistent with the hypothesis that the production of 1-hydroxyethyl radicals from ethanol by hepatic microsomes results from an attack by hydroxyl radicals generated during the microsomal oxidation of NADPH. Addition of H_2O_2 to the microsomes increased rates of hydroxyl and 1-hydroxyethyl radical production.

MATERIALS AND METHODS

Chemical agents used in these experiments were obtained from the following sources: NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, potassium azide, and aminotriazole were purchased from Sigma (St. Louis, MO). The spin trapping agent DMPO (5,5-dimethyl-1-pyrroline-N-oxide) was obtained from the

Aldrich Chemical Company (Milwaukee, WI). As received from the distributor, DMPO frequently exhibits an EPR signal by itself at the concentration employed in these experiments. Therefore, the DMPO stock solutions were routinely passed through activated charcoal columns prior to assembling the reaction systems. This procedure removes the impurities that are responsible for these signals. All other chemicals were of reagent grade quality.

Male Sprague-Dawley rats weighing 250–300 g were obtained from Sasco Corp. (Omaha, NE) and were employed as the source of liver tissue in these experiments. For experiments in which aminotriazole pretreatment was used to inhibit hepatic catalase activity (24), the animals were given an intraperitoneal injection of aminotriazole (1.0 g/kg body wt.) 1 hour prior to microsome preparation. Liver microsomes were prepared by differential centrifugation as described previously (25), with two important modifications to reduce contamination of the microsomes with catalase and other non-microsomal components. First, livers were homogenized in 10 ml of 45 mM phosphate buffer per gram of tissue in order to dilute soluable cell components. Secondly, the microsomes were washed three times to further remove contaminants (e.g., catalase, ferritin) which are loosely associated with the microsomes. When additional steps were taken to prevent contamination of the microsomes with iron, livers were perfused with chilled heparinized saline to remove red blood cells, and buffer treated with Chelex 100 resin (Bio–Rad Laboratories) was used for homogenization and all washing steps. The presence of metal ions was tested using a method based on ascorbic acid oxidation.[26] When ascorbic acid (which absorbs U.V. light at 265 nm) is dissolved in the Chelex-treated buffer, the loss of absorbance at 265 nm was less than 0.5% in 15 min, indicating that most metal contaminents had been removed by the chelation treatment.[26] The limit of sensitivity for this assay is about 0.2 μM (as EDTA-Fe^{3+}). In some experiments, the washed microsomes were further purified by chromatography on a Sepharose CL-2B column.[27] These chromatographed microsomes had a non-heme iron content averaging 2.3 nmol/mg protein, while the washed (but not chromatographed) microsomes had an average non-heme iron content of 6.6 nmol/mg protein.

The basic microsomal incubation system was composed of 40 mM DMPO, microsomes (3.0 mg protein/ml of reaction system), and 45 mM potassium phosphate buffer, pH 7.4. Reactions were started by addition of an NADPH-generating system consisting of 0.3 mM NADPH, 5 mM glucose-6-phosphate, and 0.5 Kornberg unit of glucose-6-phosphate dehydrogenase. In all systems except where specifically indicated, 100 μM azide was added to inhibit catalase which contaminates the microsomal preparations. The concentrations of other additions are indicated in the respective figure legends.

The microsomal reaction system were assembled and transferred to a quartz flat cell for direct observation of the reaction in a Bruker E-300 EPR spectrometer at room temperature. Unless otherwise noted, the EPR spectrometer settings were as follows: modulation frequency 100 kHz, modulation amplitude, 0.975 G, conversion time 327.68 ms, sweep time 335.544 s, center field 3480 G, sweep width 100 G, frequency 9.75 GHz. All spectra are accumulations of two or more scans. Hyperfine splitting constants for the spin adducts are given in the figure legends. Protein content was determined by the procedure described by Gornall *et al.*[28] The activity of catalase contaminating the microsomes was routinely assayed by the methods of Beers and Sizer.[29] Non-heme iron was measured by the method of Kok and Wild[30] using bathophenanthrolene to form a colored complex with ferrous iron. The method was

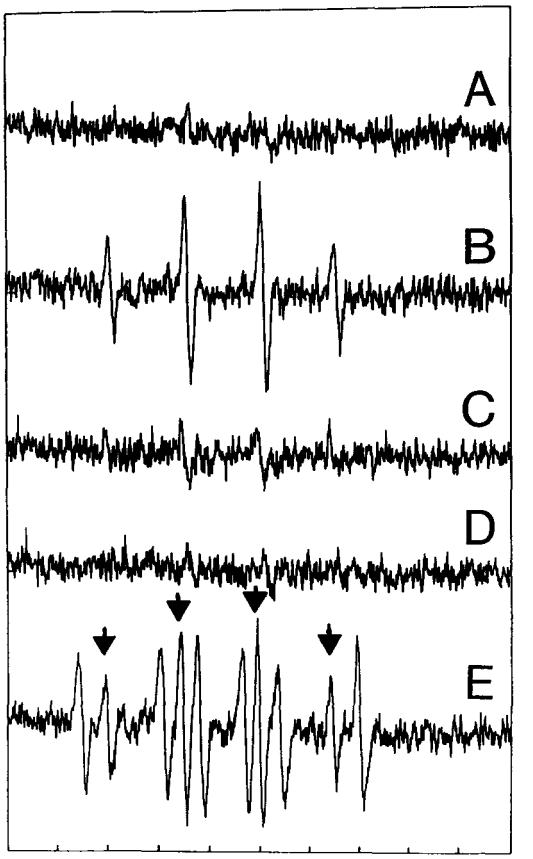

FIGURE 1 Formation of free radicals by liver microsomes during aerobic, NADPH-dependent metabolism. Hepatic microsomes were incubated with the spin trapping agent DMPO (40 mM) and an NADPH-generating system. Azide (0.1 mM) was included in spectra B-E, heat-denatured microsomes were used in spectrum C, NADPH was omitted in spectrum D, and ethanol (100 mM) was added in spectrum E. The hyperfine splitting constants measured in these microsomal systems are as follows: for the hydroxyl radical DMPO (spectra B and E), are: $a_N = a_\lambda^H = 14.9$ G; for the 1-hydroxyethyl radical adduct of DMPO (spectrum E), $a_N = 15.9$ G and $a_\beta^H = 23.1$ G. In spectrum E, the hydroxyl radical adduct peaks are indicated by the arrows. The spectra shown have a width of 100 G, with midfield of 3840 G.

slightly modified so that the volumes and concentration of reagents enabled the final volume of the assay system to be 1.1 ml instead of 4.5 ml as described by Kok and Wild.

RESULTS

When microsomes were incubated with DMPO in the presence of NADPH, no radical adducts were observed (Figure 1A). However, when azide (0.1 mM) was added to the microsomal suspension (Figure 1B), the characteristic spectrum of the hydroxyl radical adduct of DMPO was obvious. The adduct was not formed in the absence of

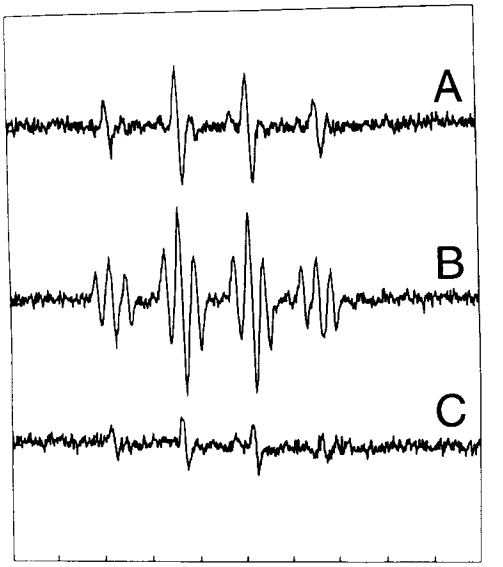

FIGURE 2 Effects of azide and aminotriazole pretreatment on microsomal formation of hydroxyl radical adducts of DMPO. Microsomes used for spectrum C were obtained from rats which had been pretreated with aminotriazole to inactivate catalase.[24] Azide was added to the microsomes in spectrum A (0.1 mM) and spectrum B (1.0 mM). The hyperfine splitting constants for the azidyl adduct of DMPO (spectrum B) were $a_N = a_\beta^H = 14.5$ G, $a_{^{15}N} = 3.2$ G, and are similar to those reported by Kalyanaraman *et al.*[31]

NADPH or when the microsomes were inactivated by prior heating (Figure 1C, 1D). When ethanol was added to the microsomal systems (Figure 1E), the EPR spectra indicated that a second adduct of DMPO was present. The identity of this second adduct was proposed to be the 1-hydroxyethyl radical, which is known to be readily trapped by DMPO.[18] This assignment was confirmed in experiments described in experiments described below in this report. Formation of the 1-hydroxyethyl adduct was also required the addition of NADPH and enzymatically active microsomes (data not shown).

Because the spectrum of the hydroxyl radical adduct of DMPO was observed only in the presence of azide, experiments were conducted to test for optimal concentrations of azide, and also to determine whether these results could be attributed to inhibition of catalase or some direct effect of azide. When the azide concentration was increased to 1.0 mM (Figure 2B), the EPR spectrum indicated the formation of the azidyl radical adduct of DMPO in addition to the hydroxyl radical adduct. These results indicate that azide can compete with DMPO and/or ethanol to scavenge hydroxyl radicals, so the concentration of azide in spin trapping experiments must be minimized. The azide concentration of 0.1 mM was found to be sufficient to cause complete inhibition of the microsomal catalase activity, but some spectra still display evidence of low concentrations of the azidyl adduct of DMPO (e.g., Figure 2A). A weak hydroxyl radical adduct could be observed in the absence of azide when microsomes from aminotriazole-treated rats were used (Figure 2C). Pretreatment of rats with

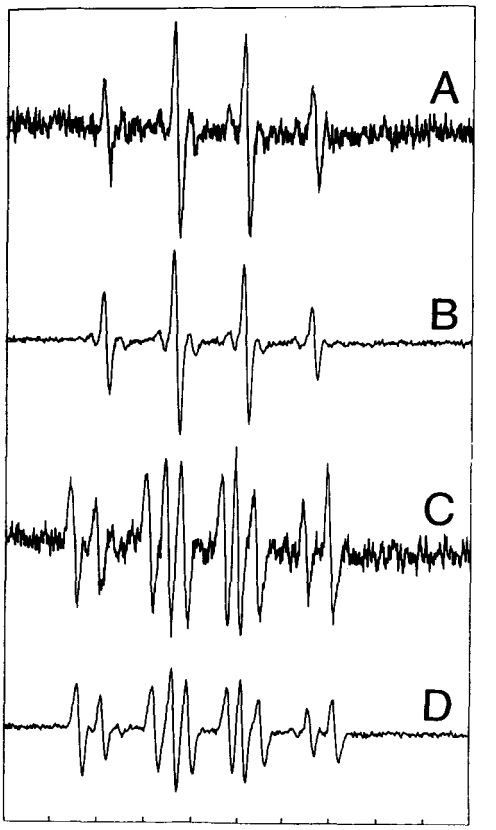

FIGURE 3 Effect of hydrogen peroxide addition on the microsomal generation of hydroxyl and 1-hydroxyethyl radical adducts of DMPO. Hepatic microsomes were incubated with azide (0.1 mM) and DMPO (40 mM). Ethanol (100 mM) was added in spectra C and D, and hydrogen peroxide (0.1 mM) was added in Spectra B and D. The spectra shown in B and D were obtained at a lower spectrometer gain setting because of a five-fold increase in signal intensity caused by hydrogen peroxide.

aminotriazole causes greater than 90% inhibition of hepatic catalase activity, as indicated previously.[24]

The addition of hydrogen peroxide to the microsomal systems increased the intensity of both the hydroxyl radical and the 1-hydroxyethyl radical adducts of DMPO (Figure 3, compare 3A with 3B and 3C with 3D). However, these DMPO adducts were not produced when NADPH was omitted, or when heat-denatured microsomes were used, even if exogenous hydrogen peroxide was added to the suspensions (data not shown). Addition of superoxide dismutase had no obvious effect on the formation of either adduct (data not shown).

The assignment of the 1-hydroxyethyl radical to the spectrum shown in Figures 1 and 3 was confirmed through the use of 1-^{13}C-ethanol (Figure 4). The ^{13}C atom splits the normal six-line spectrum into 12 lines. The spectrum observed in microsomal incubations with ^{13}C-ethanol proved to be as predicted from the hyperfine splitting

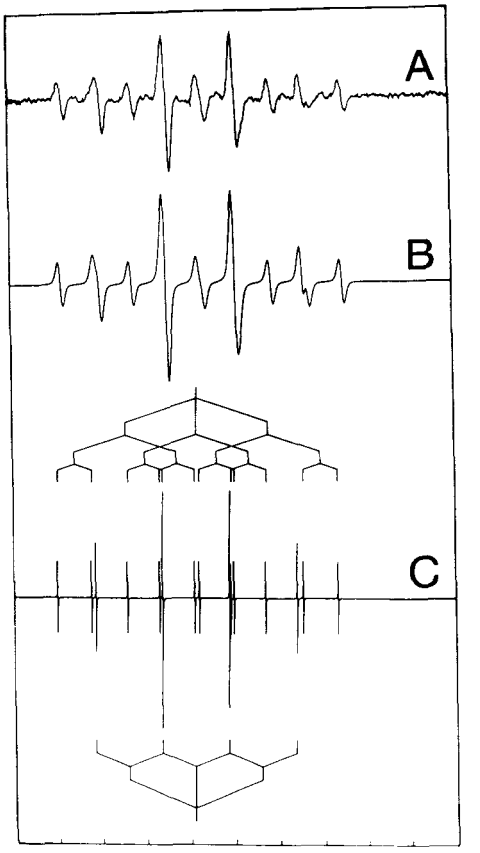

FIGURE 4 Confirmation of the generation of 1-hydroxyethyl adducts of DMPO by microsomes metabolizing ethanol. Spectrum A was obtained in the presence of 100 mM 1-^{13}C-labelled-ethanol and 0.1 mM hydrogen peroxide (A). Spectrum 2B is a computer simulation of spectrum 2A, and spectrum 2C represents the splitting pattern for the DMPO adducts of the hydroxyl (bottom) and 1-^{13}C-labeled 1-hydroxyethyl (top) radicals. Several of the spectral peaks shown in spectrum 2C were not resolved in spectra 2A and 2B. The hyperfine splitting constants of the 1-^{13}C hydroxyethyl radical adduct of DMPO in this microsomal system were a_N = 14.9 G; a_β^H = 23.1 G; $a_{^{13}C}$ = 7.8 G.

constants when overlapping spectral lines of the 1-hydroxyethyl radical and the hydroxyl radical are taken into account (Figure 4C).

When increasing concentrations of ethanol were added to the microsomal system, the formation of the 1-hydroxyethyl adducts was concomitantly increased (Figure 5). The spectral contribution of the hydroxyl radical adduct decreased as the ethanol concentration was increased, until essentially only the 1-hydroxyethyl radical adduct signal was observed at an ethanol concentration of 1.0 M (Fig. 5D).

The formation of hydroxyl radicals from hydrogen peroxide in biological systems is thought to require catalysis by iron or another redox-active metal. Therefore, the results of these studies could be explained in at least two ways. First, the hydroxyl radical formation could be associated with the microsomes *per se*. Alternatively, metal

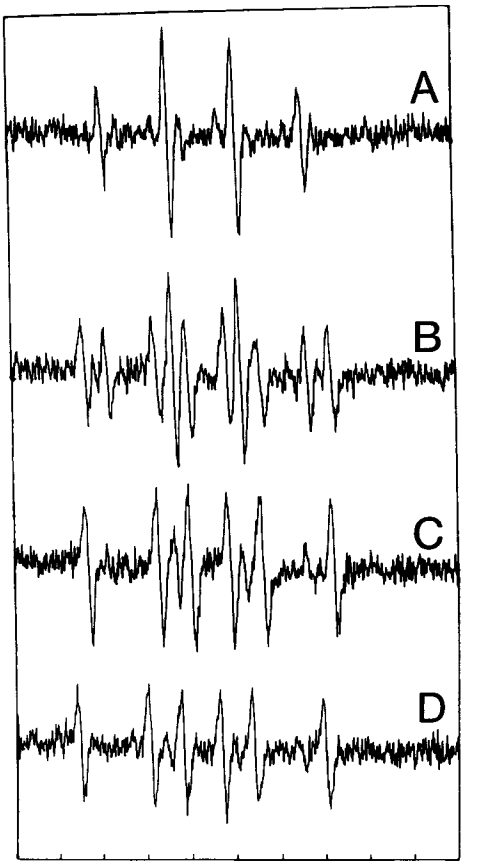

FIGURE 5 Effect of adding increasing amount of ethanol to the hydroxyl radical-generating microsomal system. A. Microsomes oxidizing NADPH in the presence of 100 μM azide (no ethanol added). The following additions were made: B. 50 mM ethanol; C. 500 mM ethanol; D. 1.0 M ethanol.

contaminants in the buffers used, together with hydrogen peroxide generated by the microsomes, could result in the artifactual formation of the hydroxyl radicals. For this reason, experiments were conducted using buffers in which the microsomes and all solutions were prepared using buffers treated with Chelex 100 resin. In addition, part of the microsomal suspension was further purified through chromatography, in order to remove ferritin as a source of iron.[27,32] The results of these experiments demonstrate that both hydroxyl and 1-hydroxyethyl radical adducts of DMPO are formed even after precautions were taken to minimize contamination by iron (Figure 6). In the more highly purified systems, weak signals were also present in addition to that of the hydroxyl radical signal (Figures 6A, 6C). These appear to be due to overlapping contributions of azidyl and superoxide adducts. Superoxide is a precursor for microsomal hydrogen peroxide, but reacts with DMPO at a much slower rate than the hydroxyl radical.[33,34] The significance of these weak signals will be the subject of a subsequent report.

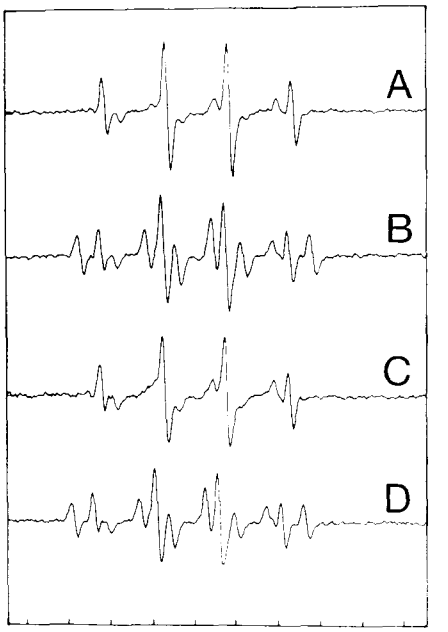

FIGURE 6 Effect of column purification of microsomes on the formation of hydroxyl and 1-hydroxyethyl radical adducts of DMPO. Hepatic microsomes were prepared with buffers treated with Chelex 100 resin (used in experiment A and B), and a portion of the microsomal suspension was further purified through chromatography on a Sepharose column (experiments C and D). All microsome proparations were ultimately suspended in KCl (0.16 M) which had been treated with Chelex 100 resin and were found to have minimal concentrations of redox-active metals in the ascorbate oxidation test.[26] Azide (0.1 mM) was added to all microsomal suspensions, and ethanol (100 mM) was added to the suspensions shown in spectra B and D. Non-chromatographed microsomes had a non-heme iron content of 6.6 nmol/mg, and catalase activity of 0.002 units/mg. Chromatographed microsomes had a non-heme iron content of 2.3 nmol/mg and catalase activity of 0.007 units/mg. The follow spectrometer conditions were employed to improve the signal-to-noise ratio: sweep time, 42 s.; conversion time, 40.96 ms.; accumulation of 16 scans.

DISCUSSION

The results of these studies clearly indicate that hydroxyl radicals are produced by liver microsomes during NADPH oxidation, and that real-time observation of hydroxyl radical generation by the microsomes depends on essentially eliminating the catalase activity which normally contaminates even repeatedly washed microsomal preparations. The presence of catalase apparently is the primary reason for the lack of any previous observation of hydroxyl radicals by microsomes using spin trapping and EPR techniques in the absence of added iron and iron chelating agents.[18–21] In addition, most studies of the metabolism of ethanol to a free radical[15–17] have employed nitrone spin traps which form unstable adducts with hydroxyl radicals.

Azide was used in most experiments to inhibit catalase, but a direct effect of azide in the formation of the radical adducts was disproven when catalase activity was minimized by aminotriazole pretreatment instead of using azide (Figure 2). In contrast, azide at high concentrations is a scavenger of hydroxyl radicals, and forms the azidyl adduct which can be trapped by DMPO (Figure 2B). Azide has been

commonly employed in studies on microsomal ethanol oxidation to inhibit the peroxidative metabolism of ethanol by hepatic microsomes.[5,6,9] Interpretation of the role of hydroxyl radicals in ethanol oxidation in such studies is complicated by two effects of azide. First, hydroxyl radical formation is intensified in the presence of azide (Figure 1), due to increasing concentrations of hydrogen peroxide resulting from the inhibition of catalase. Secondly, high concentrations of azide effectively compete with ethanol for hydroxyl radical attack (Figure 2B). Azide itself is an iron chelating compound, but we could find no evidence that in the presence of hydrogen peroxide, azide plus iron ($FeCl_{3+}$) produced hydroxyl radicals detectable by spin trapping.

The data shown in this report support the conclusion that the metabolism of ethanol to 1-hydroxyethyl radicals may be mediated at least in part by hydroxyl radicals produced in a hydrogen peroxide-dependent reaction as previously suggested in studies of microsomal oxidations[9-14] and spin trapping experiments.[16,18] The inverse relationship between the increasing signal strength for the 1-hydroxyethyl radical and the concomitant decreasing signal for the hydroxyl radical as the ethanol concentration was increased (Figure 5) is readily explained by hydrogen atom abstraction from ethanol by the hydroxyl radical. Additional support for the involvement of hydrogen peroxide in this process was obtained in experiments in which H_2O_2 was added to the reaction systems. A corresponding increase in the intensity of production of both the hydroxyl and 1-hydroxyethyl radicals was observed. In the absence of azide, ethanol may diffuse to a site of hydroxyl radical formation which is not readily accessible to DMPO, resulting in lower rates of 1-hydroxyethyl radical formation.[16,18]

In this report, the requirement for hydrogen peroxide for the production of hydroxyl radicals could be interpreted to involve a Fenton-type reaction in which the catalytic action of some form of iron is required. Experiments in which precautions were taken to prevent the introduction of adventitious iron in the buffers still demonstrated formation of hydroxyl and 1-hydroxyethyl radicals by the microsomal suspensions (Figure 6). These results indicate that the microsomes themselves provide the required catalyst, which is most likely some form of iron. Microsomes prepared through standard methods are usually contaminated with both ferritin and an unidentified form of non-heme, non-ferritin iron. Although chromatography on Sepharose columns removes most of the ferritin.[27,32] the non-ferritin iron has proven difficult to remove and continues to release low concentrations of ferrous iron under reductive conditions.[32] In the experiments described in this report, NADPH and enzymatically active microsomes were required for hydroxyl radical generation when hydrogen peroxide was generated endogenously or added to the system exogenously. These results are consistent with enzymatic reduction of some form of iron bound to the microsomes. However, the possibility that 1-hydroxyethyl radicals may be formed through hydroxy radical-independent mechanisms is not excluded. The relationships which exist among tissue iron, iron chelates and hydroxyl radical generation require additional study and can be facilitated by the spin trapping methods described in this paper.

Acknowledgements

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References

1. D. Keilin and E.F. Hartree (1945) Properties of catalase. Catalysis of coupled oxidation of alcohols. *Biochemical Journal*, **39**, 93–298.
2. A. Boveris, N. Oshino and B. Chance (1972) The cellular production of hydrogen peroxide. *Biochemical Journal*, **128**, 617–630.
3. R.N. Zahlten, M.E. Nejtek and J.C. Jacobson (1981) Ethanol metabolism in guinea pigs: *In vivo* ethanol elimination alcohol dehydrogenase distribution, and subcellular localization of acetaldehyde dehydrogenase in liver. *Archives of Biochemistry and Biophysics*, **207**, 371–379.
4. W.H. Orme-Johnson and D.M. Ziegler (1965) Alcohol mixed function oxidase activity of mammalian liver microsomes *Biochemical and Biophysical Research Communications*, **2178**, 78–82.
5. C.S. Lieber and L.M. DeCarli (1970) Hepatic microsomal ethanol-oxidizing system: *in vitro* characteristics and adaptive properties *in vivo*. *Journal of Biological Chemistry*, **254**, 2505–2512.
6. R.G. Thurman, H.G. Ley and R. Scholz (1972) Hepatic microsomal ethanol oxidation: hydrogen peroxide formation and the role of catalase. *European Journal of Biochemistry*, **25**, 420–430.
7. G.T. Miwa, W. Levin, P.E. Thomas and A.Y.H. Lu (1978) The direct oxidation of ethanol by catalase- and alcohol dehydrogenase-free reconstituted system containing cytochrom P-450. *Archive of Biochemistry and Biophysics*, **187**, 464–475.
8. D.R. Koop, E.T. Morgan, G.E. Tarr and M.J. Coon (1982) Purification and characterization of a unique isozyme of cytochrome P450 from liver microsomes of ethanol-treated rabbits. *Journal of Biological Chemistry*, **257**, 8472–8480.
9. A.I. Cederbaum, E. Dicker and E. Cohen (1980) Role of hydroxyl radicals in microsomal oxidation of alcohols. *Advances in Experimental Medicine and Biology*, **132**, 1–10.
10. A.I. Cederbaum (1987) Microsomal generation of hydroxyl radicals: its role in microsomal ethanol oxidizing system (MEOS) activity and requirement for iron. *Annals of the New York Academy of Sciences*, **492**, 35–49.
11. S. Puntarulo and A.I. Cederbaum (1988) Effect of oxygen concentration on microsomal oxidation of ethanol and generation of oxygen radicals. *Biochemical Journal*, **251**, 787–794.
12. M. Ingelman-Sundberg and A.L. Hagbjörk (1982) On the significance of the cytochrome P-450-dependent hydroxyl radical-mediated oxygenation mechanism. *Xenobiotica*, **12**, 673–686.
13. M. Ingleman-Sundberg and I. Johansson (1984) Mechanism of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450. *Journal of Biological Chemistry*, **259**, 6447–6458.
14. G. Ekström, T. Cronholm and M. Ingelman-Sundberg (1986) Hydroxyl-radical production and ethanol oxidation by liver microsomes isolated from ethanol-treated rats. *Biochemical Journal*, **233**, 755–761.
15. E. Albano, A. Tomasi, L. Goria-Gatti, G. Poli, V. Vannini and M.U. Dianzani (1987) Free radical metabolism of alcohols by rat liver microsomes. *Free Radical Research Communications*, **3**, 243–249.
16. E. Albano, A. Tomasi, L. Goria-Gatti and M.U. Dianzani (1988) Spin trapping of free radical species produced during the microsomal metabolism of ethanol. *Chemico-Biological Interactions*, **65**, 223–234.
17. L.A. Reinke, E.K. Lai, C.M. DuBose and P.B. McCay (1987) Reactive free radical generation *in vivo* in heart and liver of ethanol-fed rats: correlation with radical formation *in vitro*. *Proceedings of the National Academy of Sciences USA*, **84**, 9223–9227.
18. L.A. Reinke, J.M. Rau and P.B. McCay (1990) Possible roles of free radicals in alcoholic tissue damage. *Free Radical Research Communications*, **9**, 205–211.
19. C.-S. Lai and L.H. Piette (1978) Spin-trapping studies of hydroxyl radical production involved in lipid peroxidation. *Archives of Biochemistry and Biophysics*, **190**, 27–38.
20. A.N. Saprin and L.H. Piette (1977) Spin Trapping and its application in the study of lipid peroxidation and free radical production with liver microsomes. *Archives of Biochemistry and Biophysics*, **180**, 480–492.
21. C.-S. Lai and L.H. Piette (1977) Hydroxyl radical production involved in lipid peroxidation of rat liver microsomes. *Biochemical and Biophysical Research Communications*, **78**, 51–59.
22. A. Terano, H. Hiraishi, S. Ota, J. Shiga and T. Sugimoto (1989) Role of superoxide and hydroxyl radicals in rat gastric mucosal injury induced by ethanol. *Gastroenterology Japan*, **24**, 488–493.
23. R. Nordmann, C. Ribiere and H. Rouach (1987) Involvement of oxygen free radicals in the metabolism and toxicity of ethanol. *Progress in Clincial and Biological Research*, **241**, 201–213.
24. W.G. Heim, D. Appleman and H.T. Pyfrom (1956) Effects of 3-amino-2, 3, 4-triazole (AT) on catalase and other compounds. *American Journal of Physiology*, **186**, 19–23.

25. P.B. McCay, E.K. Lai, J.L. Poyer, C.M. DuBose and E.G. Janzen (1984) Oxygen- and carbon-centered free radical formation during carbon tetrachloride metabolism. Observation of lipid radicals *in vivo* and *in vitro*. *Journal of Biological Chemistry*, **259,** 2135–2143.
26. G. R. Buettner (1988) In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *Journal of Biochemical and Biophysical Methods*, **16,** 27–40.
27. C.E. Thomas and S.D. Aust (1984) Rat Liver Microsomal NADPH-dependent release of iron from ferritin and liped peroxidation. *Free Radicals in Biology and Medicine*, **1,** 293–300.
28. A.G. Gornall, C.J. Bardawill and M.M. David (1991) Determination of serum proteins by means of biuret reaction. *Journal of Biological Chemistry*, **177,** 751–766.
29. R.F. Beers, Jr. and I.W. Sizer (1951) A spectrophotometric method for measuring the breakdwon of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, **195,** 133–140.
30. D'A. Kok and F. Wild (1960) Serum iron determination. *Journal of Clinical Pathology*, **13,** 214–245.
31. B. Kalyanaraman, E.G. Janzen and R.P. Mason (1985) Spin trapping of the azidyl radical in azide/catalase/H202 and various azide/peroxidase/H202 peroxidizing systems. *Journal of Biological Chemistry*, **260,** 4003–4006.
32. G. Minnotti (1989) Adriamycin-dependent release of iron from microsomal membranes. *Archives of Biochemistry and Biophysics*, **268,** 398–403.
33. E.G. Janzen, D.E. Nutter, Jr., E.R. Davis, B.J. Blackburn, J.L. Poyer and P.B. McCay (1978) On spin trapping hydroxyl and hydroperoxyl radicals. *Canadian Journal of Chemistry*, **56,** 2237–2242.
34. E. Finkelstein, G.M. Rosen and E.J. Rauckman (1980) Spin trapping of superoxide and hydroxyl radical: practical aspects. *Archives of Biochemistry and Biophysics*, **200,** 1–16.

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