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1-Hydroxyethyl Radical Formation during NADPH- and NADH-Dependent Oxidation of Ethanol by Human Liver Microsomes

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SUMMARY

Ethanol can be oxidized to the 1-hydroxyethyl radical (HER) by rat and deer mice liver microsomal systems. Experiments were carried out to evaluate the ability of human liver microsomes to catalyze this reaction, compare the effectiveness of NADH with that of NADPH, and assess the possible role of cytochrome b_5 in HER formation. HER was detected as the α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone/HER adduct. Human liver microsomes catalyzed HER formation with either NADPH or NADH as cofactor; rates with NADH were ~50% those found with NADPH. Chelex-100 treatment of the reaction mixture produced marked inhibition of HER formation, suggesting that a transition metal, such as iron, was required to catalyze the reaction. The addition of ferric chloride restored HER formation. Catalase (2600 units/ml) and superoxide dismutase (500 units/ml) nearly completely inhibited the reaction with either NADPH or NADH. The NADH-dependent rates of superoxide production, detected as 5,5-dimethyl-1-pyrroline-*N*-oxide-O_2H, were ~50% the NADPH-dependent rates, which is consistent with the rates of HER formation. Anti-cytochrome b_5 IgG decreased NADPH- and NADH-dependent HER formation, and this was associated with inhibition of superoxide formation with both reductants. These results indicate that human liver microsomes can catalyze the oxidation of ethanol to HER with either NADPH or NADH as reductant. The effectiveness of NADH may be significant in view of the increased NADH/NAD^+ redox ratio in the liver as a consequence of ethanol oxidation by alcohol dehydrogenase. HER formation by human liver microsomes seems to be catalyzed by an oxidant derived from the interaction of iron with superoxide or H_2O_2, and a close association exists between HER formation and superoxide production. Cytochrome b_5 seems to play a role in HER formation, most likely due to its effect on superoxide production.

There is much interest that ethanol may promote an imbalance between prooxidant and antioxidant systems in favor of the former and that ethanol-induced oxidative stress may play a role in the liver damage produced by ethanol (1–6). Formation of reactive oxygen species by liver microsomes and their increase as a consequence of induction of P4502E1 by chronic ethanol consumption have been postulated to play a major role in ethanol-induced oxidative stress (7–11). Peroxidative damage occurs in alcoholic patients (12, 13), and increased lipid peroxidation products can be correlated with increased alcohol intake by humans (14).

Ethanol has been shown to be oxidized to a free radical metabolite, the HER, by rat (11, 15–17, 19) and deer mice (18) liver microsomal systems. HER has also been detected in rat and deer mice *in vivo* (20, 21). Covalent bound HER/protein adducts have been detected (22), and these adducts have immunological properties, leading to the formation of antibodies that can specifically recognize the HER moiety of the protein adduct (23). Such antibodies have been detected in the blood of patients with alcoholic cirrhosis (24). HER can also cause DNA/protein cross-linking (25), alkylation of DNA bases (a reaction studied with the α-hydroxyisopropyl radical) (26), and reduction in hemoproteins (27). In view of these properties, the formation of HER from ethanol may have a role in ethanol-promoted hepatotoxicity.

The mechanism of HER formation from ethanol has proved to be complex. The initial reports by Albano *et al.* (15–17) concluded that there were two pathways leading to the formation of HER from ethanol. One pathway required catalysis by iron and was inhibited by catalase; this probably represents a ·OH-dependent reaction. The other pathway was ·OH-independent and was suggested to reflect the direct one-electron oxidation of ethanol to HER by P450. Reinke *et al.* (11) also concluded that H_2O_2 played an important role in HER formation from ethanol. Knecht *et al.* (18) found that catalase had no effect on HER formation; however, SOD was nearly completely inhibitory. They suggested that ethanol is oxidized to HER by an oxidant derived from the interaction of transition metals with $O_2^{\cdot-}$ (18).

There have been few studies evaluating the production of

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ABBREVIATIONS: P450, cytochrome P450; HER, 1-hydroxyethyl radical; $O_2^{\cdot-}$, superoxide anion radical; ·OH, hydroxyl radical; POBN, α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; SOD, superoxide dismutase.

reactive oxygen intermediates by human liver microsomes; it is generally assumed that results with rat liver microsomes can be extrapolated to human liver microsomes, which may not always be true (28). In a previous study with human liver microsomes, ·OH-dependent oxidation of ethanol to HER was studied with ferric-EDTA used as an iron catalyst (28); ferric-EDTA was omitted in the current study. Most studies on microsomal production of reactive oxygen species and HER formation have used NADPH as the reductant because this is the preferred cofactor for P450-catalyzed mixed function oxidation. However, NADH was previously found to be 30–100% as effective as NADPH in catalyzing human liver microsomal production of reactive oxygen species and in reduction of ferric complexes (28). The ability of NADH to catalyze HER formation by human liver microsomes was therefore compared with that of NADPH. A final goal was to evaluate the possible role of b_5 in HER formation because this hemoprotein is involved in NADH-dependent microsomal electron transfer and is known to influence NADPH-dependent electron transfer and drug oxidation.

Materials and Methods

Rat liver microsomes were prepared from male Sprague-Dawley rats (weight, ~200 g) through standard differential centrifugation. Microsomes were isolated (29) from three human livers: a 37-year-old man with alcoholic liver disease (UN500), a 61-year-old man with alcoholic liver disease (UN501), and a 26-year-old woman with no liver pathology (UC9411). Total P450 content ranged from 0.22 to 0.24 nmol/mg microsomal protein. The human livers were obtained from The Liver Tissue Procurement and Distribution System. Cytochrome b_5 was purified from rat liver microsomes as previously described (30), and anti-b_5 IgG was prepared in rabbits with the purified b_5 used as antigen (30). Preimmune IgG was prepared from the same rabbits, with the blood removed before administration of the antigen. Western blot analysis was carried out as previously described (30). The content of P450 was determined from the carbon monoxide difference spectrum (31).

ESR measurements were performed with a Bruker ECS 106 ESR spectrometer equipped with a 4103 TM cavity and 50-KHz modulation frequency. The flat ESR cell containing the sample was placed in the cavity, and measurements were done at room temperature, routinely at an instrumental setting of microwave power of 20 MW, microwave frequency of 9.75 GHz, centerfield at 3480 G, resolution of 1024 points, sweep time of 168 sec, 5 or 10 scans, time constant of 81.9 msec/point, modulation amplitude of 2.53 (for most experiments), and scan width of 100 G. Spin-trapping of HER was performed with POBN, whereas the superoxide anion radical was spin-trapped with DMPO. For HER detection, either [1-^{13}C]ethanol or [1-^{12}C]ethanol was used. Quantification of the POBN/HER adduct was carried out by double integration of the first low-field line [$M_I(^{14}N)(^1H)(^{13}C) = (+1, +½, +½)$]. The spin concentration of DMPO-O_2H was determined by the double integration of the last high field line [$M_I(^{14}N)(^1H)_\beta(^1H)\gamma = (-1, -½, +½)(-1, -½, -½)$]. Tempol was used as a standard, and its concentration was determined with the use of the extinction coefficient of 1440 M^{-1} cm^{-1} at 240 nm (32). Specific reaction conditions are indicated in the legends to figures or tables.

Most chemicals used in these studies were purchased from Sigma Chemical Co. or Aldrich Chemical Co. Catalase, SOD, NADH, and

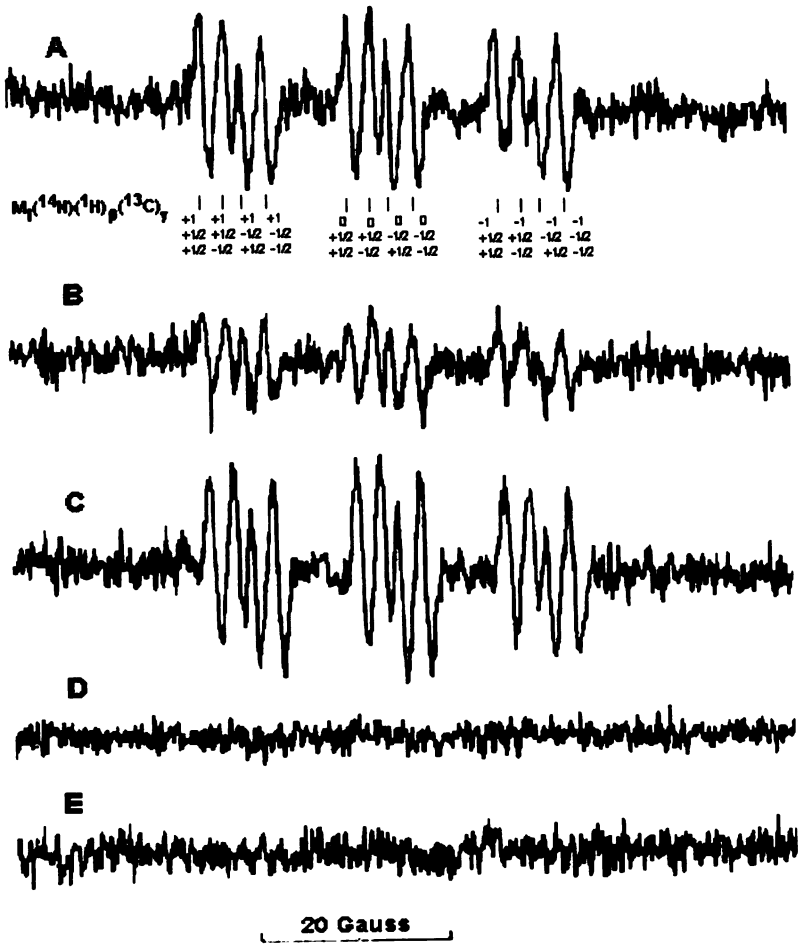

Fig. 1. ESR spectra of the POBN/HER adduct formed during the oxidation of [1-^{13}C]ethanol by human liver microsomes. Reaction mixtures contained human liver microsomes (protein equivalent to 100 pmol P450/ml), 100 mM potassium phosphate buffer, pH 7.4, 50 mM [1-^{13}C]ethanol, 20 mM POBN, 0.5 mM desferrioxamine mesylate, 0.2 mM sodium azide, and 1 mM NADPH in a final volume of 0.2 ml. Reactions were initiated with cofactor, the entire incubation mixture was placed into the flat cell, and ESR spectra were collected for various time periods, up to 2 hr, at ambient temperature. A, Control incubation. B, Same as A but with NADH (1 mM) instead of NADPH. C, NADPH plus NADH. D, Same as A but without microsomes. E, Same as A but without NADPH.

NADPH were obtained from Boehringer Mannheim Biochemicals. [1-^{13}C]Ethanol was obtained from Cambridge Isotopes, and desferrioxamine mesylate was purchased from Ciba-Geigy Ltd. Chelex-100 was obtained from Bio-Rad Laboratories. DMPO was distilled under vacuum and stored in argon at −80° before its use. POBN was prepared fresh every day.

Results

The generation of HER from [1-^{13}C]ethanol by human liver microsomes incubated with NADPH is shown in Fig. 1A. POBN was used as the trapping agent for HER, and a characteristic 12-line signal was found with [1-^{13}C]ethanol. The POBN/HER was identified by its hyperfine coupling constants: A(^{14}N) = 15.7 G, A(^{1}H) = 3.1 G, and A(^{13}C) = 2.6 G. These values, generated from the diagram shown in Fig. 1A, compare well with those reported in the literature (18, 33). The generation of HER from ethanol was also detected in the presence of NADH and human liver microsomes (Fig. 1B), with similar hyperfine coupling constants as found with NADPH. In the presence of both NADH and NADPH, the ESR signal intensity of POBN/HER was higher than that found with either NADPH or NADH alone (Fig. 1C). Desferrioxamine mesylate was present in the assay system to bind any "free" iron that may be present; sodium azide was present to inhibit any contaminating catalase activity. Formation of POBN/HER required the presence of microsomes (Fig. 1D) and reductant (Fig. 1E). H_2O_2 could not replace NADPH or NADH in supporting POBN/HER formation.

A time course for the production of POBN/HER with either NADPH or NADH is shown in Fig. 2. The formation of the adduct increased with time for a ~2-hr time period. The intensity of the POBN/HER signal found with NADH was ~50–70% that found with NADPH (NADPH/NADH ratio = 1.79 ± 0.06, nine experiments).

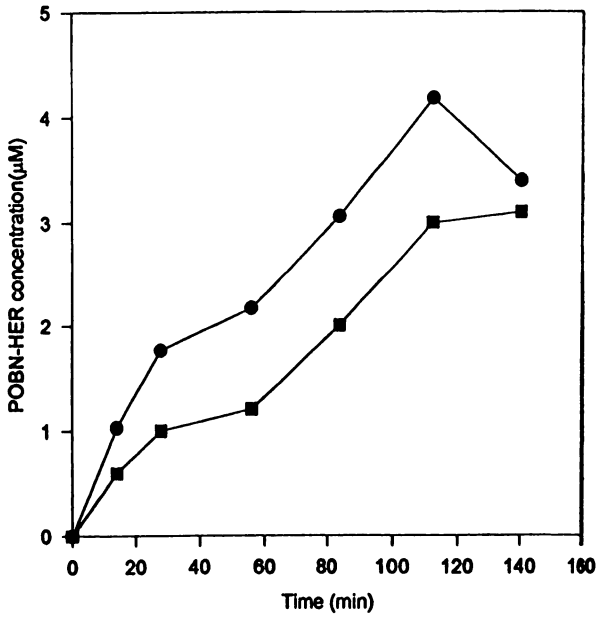

Fig. 2. Formation of POBN/HER during the oxidation of [1-^{13}C]ethanol by human liver microsomes as a function of time. The concentration of the POBN/HER adduct was determined as described in Materials and Methods. The reaction conditions are the same as described in the legend to Fig. 1; results are from data obtained after 10 scans. ●, With NADPH (1 mM). ■, With NADH (1 mM).

To evaluate whether HER formation occurs via direct oxidation by P450 or from reactive oxygen intermediates such as $O_2^{\cdot-}$ or H_2O_2 generated during microsomal electron transfer, the effects of SOD or catalase were studied. Results were similar with either NADPH or NADH; SOD (500 units/ml) completely inhibited the formation of HER (Fig. 3, B and F). Low concentrations of catalase had no effect on HER formation (Fig. 3, C and G; see legend), whereas high concentrations of catalase (2600 units/ml) completely inhibited HER formation (Fig. 3, D and H). The inhibition by catalase was prevented with azide, indicating that inhibition was dependent on catalytic activity of catalase. Because experiments were carried out in the presence of a high concentration of desferrioxamine, it was thought that little or no iron would be available to interact with $O_2^{\cdot-}$ or H_2O_2 to produce more potent oxidants that could oxidize ethanol to HER. However, treatment of the entire reaction system with Chelex-100 resin produced strong inhibition of the POBN/HER signal (Fig. 4C). That the inhibition by treatment with Chelex-100 resin seemed to reflect removal of iron was shown by the ability to restore POBN/HER formation through the addition of ferric-chloride. As "little" as 1 μM $FeCl_3$ restored POBN/HER formation to the intensity found in the absence of Chelex treatment (Table 1). Small increases in POBN/HER formation were observed on the increased addition of $FeCl_3$ (Table 1).

The above experiments were repeated with hepatic microsomes from untreated rats as the enzyme source to assess whether ethanol oxidation to HER by rat liver microsomes exhibited the same requirements as those found with human liver microsomes and those reported previously with liver microsomes from alcohol dehydrogenase-deficient deer mice (18). It was found that rat liver microsomes generated POBN/HER adducts with either NADPH or NADH as cofactor, although NADPH was ~3-fold more effective than NADH in supporting this reaction. SOD completely inhibited HER formation by rat liver microsomes with either NADPH or NADH.

The potent inhibition of microsomal HER formation from ethanol by SOD indicated that $O_2^{\cdot-}$ played a role in forming an oxidant capable of oxidizing ethanol to HER. Therefore, we determined the production of $O_2^{\cdot-}$ by human liver microsomes with DMPO used as the spin-trapping agent. These reactions were carried out in the presence of ethanol to allow estimation of $O_2^{\cdot-}$ production under conditions where HER formation also occurred. The $O_2^{\cdot-}$/DMPO adduct, DMPO-OOH, was detected when microsomes were incubated with either NADPH (Fig. 5A) or NADH (Fig. 5B). In addition, DMPO-HER and small amounts of DMPO-OH were observed (Fig. 5). The effectiveness of NADH in supporting $O_2^{\cdot-}$ production by human liver microsomes was approximately one half of that observed with NADPH.

The first electron supplied to the P450 monooxygenase system is derived from NADPH/P450 reductase, whereas the second electron may be supplied by b_5 (34–39). NADH-dependent monooxygenation reactions require electron transfer from NADH/b_5 reductase to b_5 to P450 (30, 36, 39), as does NADH stimulation of NADPH-dependent drug oxidation (34, 37–39). Therefore, b_5 may participate not only in electron transfer from NADH and NADPH but also in drug oxidations dependent on these cofactors. The possible role of b_5 in HER formation by human and rat liver microsomes was assessed

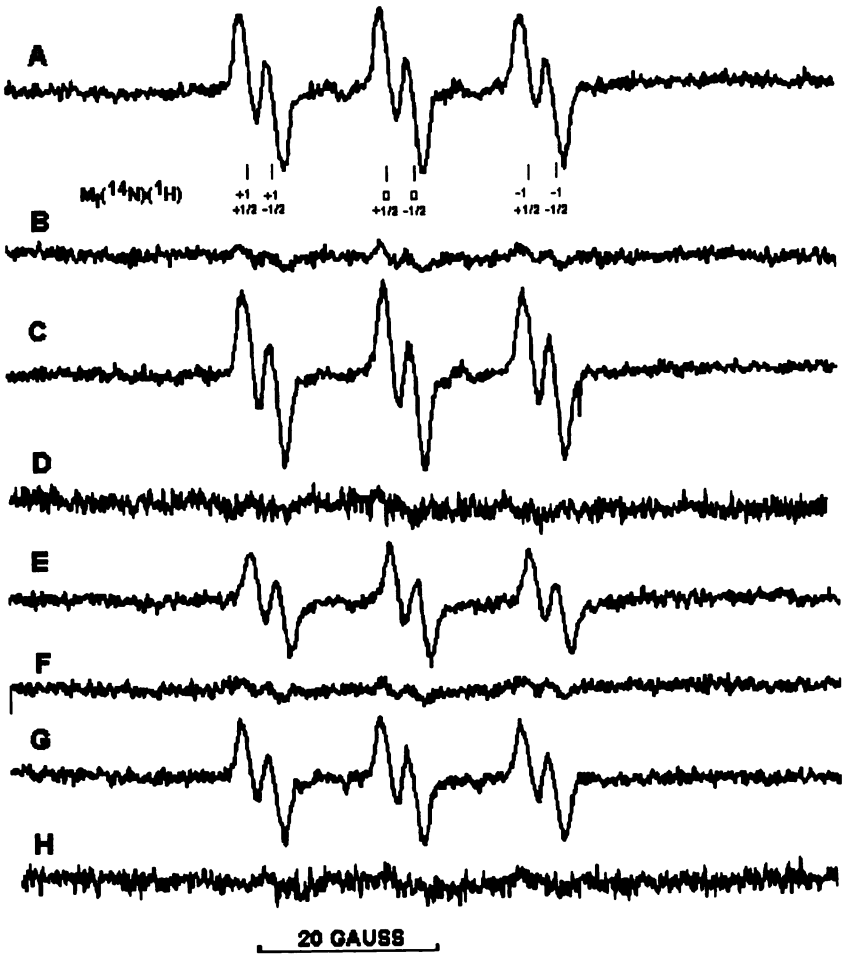

Fig. 3. ESR spectra of the POBN/HER adduct formed during the oxidation of ethanol by human liver microsomes. Reaction mixtures contained the same components as described in the legend for Fig. 1 except that [1-^{12}C]ethanol was used instead of [1-^{13}C]ethanol. Modulation amplitude was 2.57. A, NADPH (1 mM) control. B, Same as A but with SOD (500 units). C, Same as A but with catalase (130 units) and without sodium azide. D, Same as A but with catalase (2600 units) and without sodium azide. E, NADH (1 mM) control. F, Same as E but with SOD (500 units). G, Same as E but with catalase (130 units) and without sodium azide. H, Same as E but with catalase (2600 units) and without sodium azide. Catalase at concentrations of 260, 390, 650, and 1300 units/ml did not significantly affect HER formation (spectra not shown but similar to C and G).

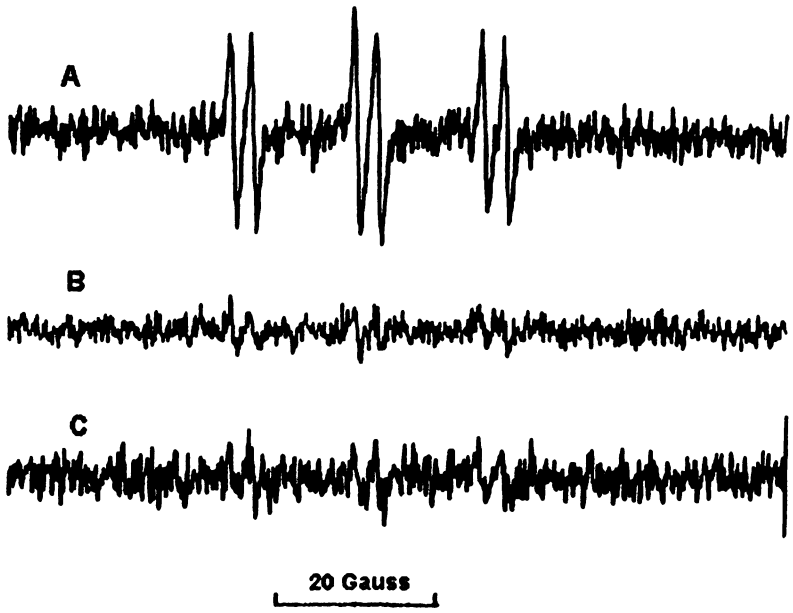

Fig. 4. A, ESR spectra of POBN/HER formed during the oxidation of [1-^{12}C]ethanol by human liver microsomes. Reaction mixtures contained the same components as given in the legend for Fig. 1A. Modulation amplitude was 1.27 G. B, Same as A, but the incubation mixture was heated at 100° for 15 min before the addition of NADPH. C, Same as A, but the incubation mixture was treated with Chelex-100. Complete reaction mixtures were swirled in tubes with Chelex-100 resin, and aliquots were withdrawn and immediately placed into the flat cell. Further details are given in the legend to Table 1.

with a highly specific inhibitor, anti-b_5 IgG. The addition of anti-b_5 IgG to rat liver microsomes failed to inhibit NADH/ferricyanide or NADPH/cytochrome *c* reductase activity but did inhibit NADH/cytochrome *c* reductase activity, indicating that the antibody was capable of blocking electron transfer to or from b_5 (30). The b_5 antibody elicited the same effects on its addition to human liver microsomes; NADPH/cytochrome *c* reductase was minimally inhibited (10%), whereas NADH/cytochrome *c* reductase was inhibited 50% at an anti-b_5 IgG/microsomal protein ratio of 5:1 mg/mg. Western blot analysis with the antibody indicated that it recognized only a single band in human liver microsomes with the same mobility as

TABLE 1

Effect of Chelex-100 resin and $FeCl_3$ on the formation of POBN/HER by human liver microsomes

Formation of POBN/HER was assayed as described in the legend to Fig. 1 with 1 mM NADPH as a cofactor with human liver microsomes (equivalent to 200 pmol/ml total P450). POBN/HER detected in the absence of Chelex-100 treatment is taken as the 100% value. Experiments were carried out by mixing the components of the incubation mixture in a microfuge tube containing half of the equivalent amount of chelex (v/v; previously washed liberally with water followed by the buffer and supernatant completely aspirated). The sample was swirled for a few seconds, pH was measured (7.4), and the sample was allowed to stand for 1 or 2 min. The supernatant was aspirated into the ESR flat cell, and ESR measurements were performed in the absence or presence of the indicated concentrations of added $FeCl_3$. The concentration of POBN/HER ranged from 0.02 to 0.32 μM.

Concentration of $FeCl_3$ added after Chelex treatment	POBN/HER detected
μM	%
0.00	10
0.25	67
0.50	74
1.00	105
1.50	150
2.00	155
3.00	160

purified cytochrome b_5 (Fig. 6, compare *lanes 1 and 4* with *lanes 3 and 6*). Anti-b_5 IgG inhibited NADH- as well as NADPH-dependent formation of HER by the human liver microsomes in a concentration-dependent manner, whereas preimmune IgG had little or no effect (Table 2). In a similar manner, the anti-b_5 IgG also produced inhibition of $O_2^{\cdot-}$ formation (DMPO-O_2H formation) by human liver and rat liver microsomes with either NADPH or NADH as cofactors (Table 3). The anti-b_5 IgG did not inhibit $O_2^{\cdot-}$ formation by a nonmicrosomal system, e.g., the xanthine oxidase reaction (data not shown).

The ability of anti-b_5 IgG to inhibit $O_2^{\cdot-}$ production by human liver microsomes was also assessed through examination of the effects of the antibody on SOD-sensitive rates of acetylated cytochrome *c* reduction. At anti-b_5 IgG/microsomal protein ratios of 10:1 mg/mg, anti-b_5 IgG inhibited both NADH- and NADPH-dependent, SOD-sensitive reduction of acetylated cytochrome *c* (Table 4). Preimmune IgG had no effect on acetylated cytochrome *c* reduction (Table 4).

Discussion

Studies by Albano *et al.* (15–17, 40), Knecht *et al.* (18, 20), and Reinke *et al.* (4, 11, 19, 21) have shown that rat and deer mice liver microsomes can catalyze the one-electron oxidation of ethanol to HER via a variety of mechanisms. Ethanol can be oxidized to acetaldehyde through a two-electron oxidation process; this pathway is in general independent of reactive oxygen species and reflects the direct oxidation of ethanol by P450 (41, 42). HER formation from ethanol, regardless of its mechanism, is therefore only a minor pathway of ethanol oxidation by microsomes but is likely to be of toxicological significance (20–24, 43).

In the current report, human liver microsomes were shown to be capable of also oxidizing ethanol to HER. Reducing equivalents are required for this reaction, and NADH has been found to be approximately one half as effective as NADPH in supporting this reaction with human liver microsomes and ~30% as effective as NADPH with rat liver microsomes. The reaction seems to require catalysis by transition metals because Chelex-100 treatment of the incubation medium markedly suppressed HER formation with both cofactors via a reaction reversible by the addition of $FeCl_3$. The requirement for iron seems to be low because as little as 1 μM additional iron restores HER formation to control rates found in the absence of Chelex treatment. Experiments were carried out in the presence of desferrioxamine to mimic the conditions reported by Knecht *et al.* (18) in an attempt to remove as much free iron from the reaction system as possible, thereby allowing evaluation of direct oxidation of ethanol to HER. These results confirm (18) the inability of desferrioxamine to completely inhibit HER formation from ethanol by a reaction that still seems to require an iron catalyst.

$O_2^{\cdot-}$ and H_2O_2 play roles in HER formation by human liver microsomes with either NADPH or NADH based on the potent inhibition by SOD or catalase. Hydroxyl radical produced by Fenton or Haber-Weiss types of reactions or other oxidants derived from the interaction of nonheme iron with either $O_2^{\cdot-}$ or H_2O_2 seem to play a significant role in HER formation under these reaction conditions. Reaction conditions that stimulate ·OH formation will stimulate HER formation from ethanol, e.g., previous experiments with human liver (28) or rat liver (44) microsomes utilized ferric-EDTA as the iron catalyst. Ferric-EDTA markedly stimulates microsomal ·OH formation (45), and under these conditions, HER formation is inhibited by catalase and competitive ·OH scavengers (28, 44). The requirement for $O_2^{\cdot-}$ and H_2O_2 in the formation of the oxidant responsible for HER formation by human liver microsomes and the sensitivity to SOD and catalase suggests that there is little or no role for a pathway involving direct oxidation of ethanol to HER by P450 under these reaction conditions, at least with POBN as the spin-trapping agent. In microsomal systems, most of the $O_2^{\cdot-}$ and H_2O_2 is produced via decay of oxygenated P450 (46, 47). Inhibitors of P450 function that prevent HER formation from ethanol most likely do so because they also prevent $O_2^{\cdot-}$ and H_2O_2 production.

Cytochrome b_5 can influence P450-catalyzed oxidative reactions (34–39). Cytochrome b_5 can donate the second electron to oxygenated P450 (34–39); this may or may not influence overall monooxygenase activity. In addition to the product, oxidation of substrates by P450 produces $O_2^{\cdot-}$, H_2O_2, and H_2O (48), and b_5 may influence the balance in the ratio of these products. The addition of b_5 was found to enhance product formation while decreasing H_2O_2 production with P4502B1; i.e., b_5 increased coupling of the system (49). Pompon (50) and Gorsky and Coon (51) reported that in the absence of a substrate for P450LM2, b_5 slightly decreased H_2O_2 formation without an effect on NADPH oxidation; in the presence of an effective substrate, b_5 decreased H_2O_2 formation but not NADPH oxidation. Cytochrome b_5 had no effect on activity of P450LM4 (51). The nature of the substrate used had a marked influence on the consumption of excess NADPH and O_2, and in contrast to results with P450LM2, b_5 had no effect on the proportion of excess NADPH and O_2 with P450RLM2 and P450RLM5 (52). With P4502E1, the addition of b_5 caused a large decrease in H_2O_2 formation and NADPH oxidation, in the absence or presence of a substrate, and it was concluded that with this isoform, b_5 increases electron flow for substrate hydroxylation at the expense of H_2O_2 and H_2O formation (53). Thus, it seems that the influence of b_5 on stoichiometry of P450-catalyzed reac-

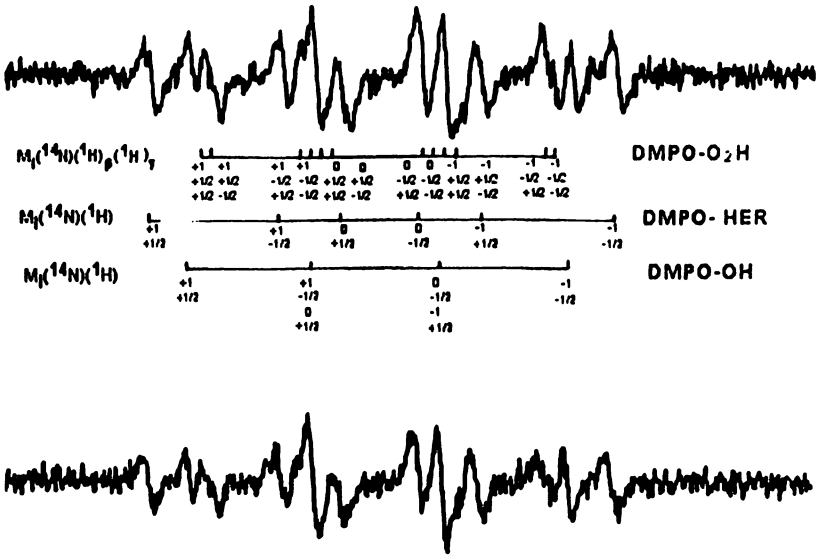

Fig. 5. *Top*, ESR spectra of DMPO-spin adducts formed during the microsomal oxidation of [1-^{12}C]ethanol at pH 6.5. Reaction mixtures contained 0.5 mM desferrioxamine mesylate, 65 units catalase, 120 mM [1-^{12}C]ethanol, 1 mM NADPH, human liver microsomes (~1.8 mg protein/ml, equivalent to ~400 pmol total P450/ml), and 90 mM DMPO in a final volume of 0.2 ml. Reactions were initiated with cofactor, and ESR spectra were recorded for various time points at room temperature. *Bottom*, same as top but with NADH (1 mM) instead of NADPH.

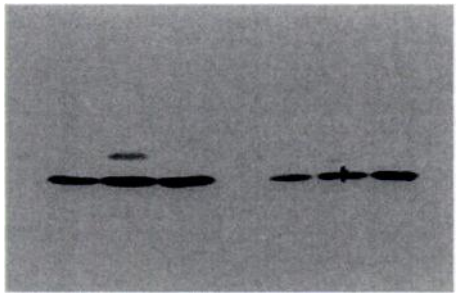

Fig. 6. Western blot analysis for the detection of cytochrome b_5 with polyclonal anti-b_5 IgG. Immunoblotting was carried out as described previously (30). *Lane 1*, 10 μg human liver microsomes; *lane 2*, 10 μg of rat liver microsomes; *lane 3*, 2 μg of purified cytochrome b_5 (specific content, 50 nmol/mg protein); *lane 4*, 5 μg of human liver microsomes; *lane 5*, 5 μg of rat liver microsomes; *lane 6*, 1 μg of purified cytochrome b_5.

TABLE 2

Effect of anti-b_5 IgG and preimmune IgG on HER formation by human liver microsomes

Formation of POBN/HER was assayed as described in the legend to Fig. 1 with either 1 mM NADPH or 1 mM NADH as cofactor. Microsomes were preincubated with anti-b_5 IgG or preimmune IgG for 3 min at 37° before the other additions. Control values in the absence of IgG were NADPH, 167 ± 13; NADH, 96 ± 6 nM POBN/HER.

Ratio of mg IgG/mg microsomal protein	Inhibition of POBN/HER formation			
	Anti-b_5 IgG		Preimmune IgG	
	NADPH	NADH	NADPH	NADH
	%			
2:1	20	63	4	1
5:1	48	73	16	5
10:1	67	82	8	−6

tions is complex and dependent on the isoform of P450, absence or presence of substrate, and the nature of the substrate. Anti-b_5 IgG produced inhibition of HER formation, suggesting that b_5 is coupled to P450 in the overall pathway

TABLE 3

Effect of anti-b_5 IgG on $O_2^{\cdot-}$ formation by human and rat liver microsomes

Formation of DMPO-O_2H was assayed as described in the legend to Fig. 5 with either 1 mM NADPH or 1 mM NADH as cofactor and either human liver or rat liver microsomes. Anti-b_5 (and preimmune) IgG was preincubated with the microsomes for 3 min at 37° before the addition of the other components of the reaction system. Preimmune IgG produced 3–10% inhibition of DMPO-O_2H formation.

Ratio of mg IgG/mg microsomal protein	Inhibition of DMPO-O_2H formation			
	Human microsomes		Rat microsomes	
	NADPH	NADH	NADPH	NADH
	%			
2:1	35	34	32	42
5:1	44	51	40	55

of HER formation. This could reflect a role for b_5 in enhancing $O_2^{\cdot-}$ production; indeed, anti-b_5 IgG inhibited $O_2^{\cdot-}$ production with both NADPH and NADH. Inhibition with NADH was expected (see below); however, inhibition with NADPH was surprising because it suggests that electron flow through b_5 promotes $O_2^{\cdot-}$ production and, subsequently, HER formation. A scheme summarizing these results is shown in Fig. 7. P450 may be reduced directly with NADPH/P450 reductase (rate constant, 1.1 S^{-1}) (39), as well as with reduced cytochrome b_5 (rate constant, 0.47 S^{-1}) (39). Inhibition of the latter pathway by anti-b_5 IgG may decrease formation of $O_2^{\cdot-}$ and H_2O_2 and, subsequently, HER production. Specificity of the anti-b_5 IgG was determined through observation of inhibition of NADH but not NADPH/cytochrome *c* reductase activity, through interaction with only one protein as detected with Western blots, and through lack of inhibition of $O_2^{\cdot-}$ produced by xanthine oxidase. Further studies with human liver microsomes and reconstituted systems with purified human P450 isoforms are in progress to evaluate the influence of b_5 on $O_2^{\cdot-}$, H_2O_2, and HER formation.

NADH was found to catalyze HER formation from the oxidation of ethanol by human and rat liver microsomes. The NADH-dependent reaction, similar to the NADPH-depen-

TABLE 4

Effect of anti-b_5 IgG on SOD-sensitive reduction of acetylated cytochrome *c* by human liver microsomes

The reduction of acetylated cytochrome *c* was determined by following the increase in absorbance at 550 nm as a function of time in the presence of either NADPH or NADH and the indicated concentrations of preimmune or anti-b_5 IgG. Rates of reduction were determined in the absence and presence of 50 units SOD/ml, and results refer to the SOD-sensitive rate of acetylated cytochrome *c* reduction. Numbers in parentheses refer to rates (nmol/min/mg microsomal protein) of SOD-sensitive acetylated cytochrome *c* reduction for the NADPH- and NADH-dependent reactions. Reaction mixtures contained human liver microsomes (protein equivalent to 100 pmol P450), 100 mM potassium phosphate, pH 7.4, buffer, 1 mM NADPH, 0.05 mM acetylated cytochrome *c*, and the indicated amounts of preimmune or anti-b_5 IgG.

Ratio of mg IgG/mg microsomal protein		Effect on acetylated cytochrome *c* reduction	
		NADPH	NADH
		%	
	0	(0.18 ± 0.01)	(0.112 ± 0.02)
Preimmune	2:1	+5	+10
	5:1	+5	0
	10:1		−10
Anti-b_5	2:1	−27	−48
	5:1	−59	−48
	10:1		−70

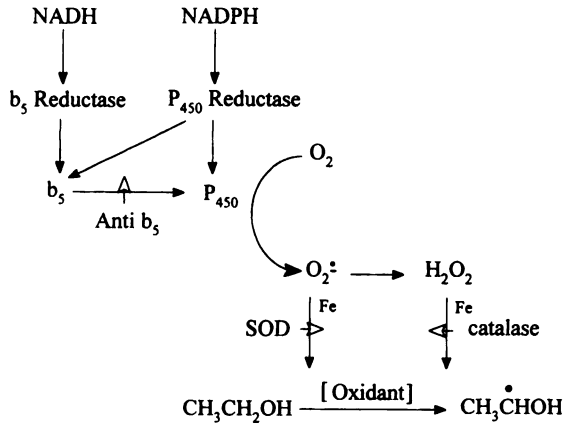

Fig. 7. Scheme for the formation of HER from ethanol during NADPH- and NADH-dependent human microsomal electron transfer.

dent reaction, was sensitive to SOD and catalase, to Chelex-100 treatment, and to anti-b_5 IgG. With human liver microsomes, NADH-dependent HER formation was ~50% the NADPH-dependent reaction; with control rat liver microsomes, the NADH-dependent rate was ~30% the NADPH-dependent reaction. It is not known whether induction of certain P450 isozymes would change the relative effectiveness of NADH versus NADPH. The relative effectiveness of NADH versus NADPH in supporting HER formation seems to reflect their effectiveness in supporting $O_2^{\cdot-}$ production. The ability of NADH to effectively catalyze HER formation may be especially significant as one of the more notable effects of ethanol on liver function is an increase in the cellular NADH/NAD^+ redox ratio as a consequence of the oxidation of ethanol by alcohol dehydrogenase (54, 55). Consequently, it may be interesting to speculate that ethanol-derived NADH from the alcohol dehydrogenase reaction provides a supply of reductant for HER formation by a microsomal-derived oxidant.

Because transfer of electrons from NADH eventually to P450 involves cytochrome b_5 (Fig. 7), the inhibition of NADH-dependent HER formation by anti-b_5 IgG may be due to antibody inhibition of iron reduction by b_5 and inhibition of $O_2^{\cdot-}$ production due to prevention of electron transfer to P450 via b_5. Indeed, the inhibition of NADH-dependent HER formation is accompanied by a parallel inhibition of $O_2^{\cdot-}$ production by anti-b_5 IgG.

The requirement for iron and the inhibition by SOD and catalase suggest that oxidants derived from the interaction of iron with $O_2^{\cdot-}$ or H_2O_2 play a major role in the one-electron oxidation of ethanol to HER. Our laboratory has previously shown that the microsomal oxidation of glycerol and other vicinal polyhydroxylated alcohols to formaldehyde plus a product with one less carbon atom was catalyzed by an oxidant derived from the interaction of iron with H_2O_2 (56). Formaldehyde production from glycerol was inhibited by iron chelators and by catalase but was not sensitive to SOD or to hydroxyl radical scavengers (56). This reaction was inhibited by P450 inhibitors and anti-P450 IgG, indicating the requirement for P450 activity (57). It was suggested that the P450 functioned to reduce iron and to generate the H_2O_2 (most likely from $O_2^{\cdot-}$ dismutation) required to produce the oxidant responsible for glycerol oxidation to formaldehyde and that glycerol was an indirect substrate for an oxidant derived from P450 electron transfer (56, 57). It seems that ethanol also functions as an indirect substrate for an oxidant derived from P450 activity when it is oxidized to HER. Production of $O_2^{\cdot-}$ and H_2O_2 during NADPH- (and NADH-) dependent electron transfer and subsequent interaction of these compounds with iron give rise to oxidants that can oxidize ethanol to HER or oxidize glycerol to formaldehyde.

In summary, human liver microsomes have been shown to catalyze the formation of HER from ethanol. Although NADPH is the preferred reductant for this reaction, NADH is also effective as a cofactor for HER formation, which may be significant in view of the ability of ethanol oxidation to generate NADH. The powerful inhibition by SOD and catalase suggests that HER formation by human liver microsomes is catalyzed by oxidants derived from the interaction of iron with $O_2^{\cdot-}$ or H_2O_2 and that direct oxidation of ethanol to HER by P450 does not play a major role under these conditions. Cytochrome b_5 seems to play a role in promoting HER formation because anti-b_5 IgG produces inhibition with either NADPH or NADH. This inhibition by anti-b_5 IgG of HER formation seems to reflect the inhibition of $O_2^{\cdot-}$ production by the antibody.

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