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Increased NADPH- and NADH-Dependent Production of Superoxide and Hydroxyl Radical by Microsomes after Chronic Ethanol Treatment

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There is increasing evidence that elevated production of reactive oxygen intermediates may contribute to the hepatotoxic actions of ethanol. Microsomes from ethanol-treated rats have been shown to generate oxygen radicals at elevated rates. Most of these studies have utilized chemical analysis techniques to assay for the production of reactive oxygen intermediates. Experiments employing the spin-trapping ESR spectroscopy technique were carried out for a more definitive characterization of production of reactive oxygen intermediates such as superoxide and hydroxyl radical (·OH) by microsomes from ethanol-fed rats and pair-fed controls, in the presence of either NADPH or NADH as the microsomal reductant. Superoxide production was determined by assaying the superoxide dismutase-sensitive generation of stable nitroxyl radical from 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine resulting from hydrogen abstraction by superoxide radical, while ·OH production was determined by assaying the formation of the POBN-α-hydroxyethyl radical adduct resulting from addition of the hydroxyethyl radical produced by hydrogen abstraction by ·OH. Microsomes from ethanol-treated rats displayed elevated rates of superoxide production with NADPH (50%) as well as with NADH (34%). With both microsomal preparations, the NADPH-dependent rates were three- to fourfold greater than the NADH-dependent rates. Microsomes from the ethanol-treated rats also displayed elevated rates of ·OH production (POBN–HER adduct formation) with NADPH (74%) and NADH (52%). With both microsomal preparations, NADPH-dependent rates were equivalent to the rates with NADH. The increase in superoxide and ·OH production after ethanol treatment was highly significant ($n = 6$, $P < 0.001$). Production of the POBN–HER adduct was sensitive to catalase, mannitol, and GSH, but not to superoxide dismutase. Addition of desferrioxamine caused a marked decrease in the signal intensity; the POBN–HER adduct formed under these conditions most likely reflects the previously reported radical-independent, direct oxidation of ethanol by cytochrome P450. Signal intensity in the presence of desferrioxamine was about threefold higher after ethanol treatment. These results demonstrate that production of reactive oxygen intermediates by microsomes, as determined by ESR spectroscopy, is elevated after chronic ethanol consumption, and that increases in production of reactive oxygen intermediates can be found with NADPH as well as NADH as the microsomal reductant.

There is increasing evidence and interest that ethanol toxicity may be associated with elevated production of reactive oxygen intermediates by the liver. DiLuzio and co-workers showed that ethanol could enhance lipid peroxidation in the liver and anti-oxidants could prevent the ethanol-induced fatty liver (1, 2). However, the ability of ethanol to promote lipid peroxidation is still controversial (3), most likely reflecting numerous variables in the models and reaction conditions used and the parameters measured (4). A variety of mechanisms exist by which ethanol, either directly or via its metabolism, can produce oxidative stress, and these models have been reviewed elsewhere (3–6).

During NADPH-dependent electron transfer isolated microsomes generate superoxide and H_2O_2 (7–14), both of which, in the presence of iron, can produce other reactive oxygen species such as the hydroxyl radical (·OH)[2]

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[2] Abbreviations used: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; $O_2^{\cdot-}$, superoxide anion radical; POBN, α-[4-pyridyl-1-oxide]-*N*-tert-butylnitrone; HER, hydroxyethyl radical; DMSO, dimethyl sulfoxide; KMB, 2-keto-4-thiomethylbutyric acid.

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and other oxidants capable of promoting lipid peroxidation (15–17). Microsomes isolated from rats treated chronically with ethanol were shown by chemical methods to have elevated rates of $O_2^{\cdot-}$ and H_2O_2 production (18–21). Spectroscopic determination of $O_2^{\cdot-}$ by ESR methods after ethanol treatment has not been reported. In the presence of the appropriate iron chelate, microsomes from ethanol-treated rats were more reactive than pair-fed controls in lipid peroxidation, chemiluminescence, and inactivating added metabolic enzymes (19, 22–24). Increased generation of ·OH-like species by microsomes after ethanol treatment has been observed in several laboratories (25–28). In all these reports, ·OH was detected via the oxidation of chemical scavengers, notably ethylene production from 2-keto-4-thiomethyl-butyric acid and formaldehyde production from DMSO. Increased oxygen radical production by microsomes after chronic ethanol treatment may be due to increased content of mixed-function oxidase enzymes (NADPH-cytochrome P450 reductase, total cytochrome P450), induction of cytochrome P4502E1, inefficient coupling between the reductase and P450, and/or increased oxidase activity of the P4502E1 isozyme (19, 29).

Most studies on the generation of reactive oxygen intermediates by microsomes have utilized NADPH as the microsomal reductant. All the above studies dealing with the effects of chronic ethanol treatment utilized NADPH. NADH was recently shown to be as reactive as NADPH in reducing various ferric chelates and at least 50% as reactive as NADPH in catalyzing production of ·OH-like species (30). With microsomes from ethanol-treated rats, rates of H_2O_2 and ·OH production were elevated about 50% with NADH as the cofactor; microsomal lipid peroxidation assessed as the production of thiobarbituric acid-reactive components was increased 60% with NADH as reductant, compared to a 120% increase with NADPH (31). Thus with either NADPH or NADH, microsomes from ethanol-treated rats generate increased amounts of reactive oxygen species. More specific and direct methods for the determination of ·OH and other oxidizing radical species such as ESR spectroscopy have not been carried out with NADH as the microsomal reductant.

Recent ESR spectroscopy experiments by Albano *et al.* (32, 33) and Reinke *et al.* (34, 35) have shown the production of the α-hydroxyethyl radical (HER) when ethanol was incubated with NADPH and microsomes. Two pathways were suggested to participate in the production of HER, one dependent on oxygen radicals, the other, independent of oxygen radicals. The latter pathway appears to be mediated by direct oxidation of ethanol by cytochrome P450, especially P4502E1 (32, 34). Concerning the oxygen radical-dependent pathway, a critical role for iron was implicated based upon inhibition of the ESR signal by added desferrioxamine and augmentation of the signal by added iron (32, 34). It is unclear if ·OH or some other oxidant produced from interaction of iron with H_2O_2 is involved in generating HER. Knecht *et al.* (36) observed HER in the bile of deermice lacking alcohol dehydrogenase, demonstrating production of this radical by intact cells.

The goal of the current report was to employ ESR spectroscopy for the more direct determination of radicals such as $O_2^{\cdot-}$ and ·OH by microsomes from ethanol-treated rats and pair-fed controls. Reaction conditions, as described in the preceding manuscript (37), were developed to allow suitable kinetics of the reaction to be followed, thereby allowing a more direct and sensitive approach for comparing rates of oxygen radical production with NADH or NADPH by both types of microsomal preparations.

MATERIALS AND METHODS

Male, Sprague–Dawley rats with starting weights of 110–120 g were fed for 6–8 weeks with the Lieber–DeCarli liquid diet in which ethanol provided 36% of total calories, protein 18%, fat 35%, and carbohydrate 11% (38). Pair-fed littermates consumed the same diet except that carbohydrates isocalorically replaced ethanol. Liver microsomes were prepared as described previously, washed once, suspended in 125 mM KCl, and stored at −80°C. Protein concentration was determined by the method of Lowry *et al.* (39) and the content of cytochrome P450 by the method of Omura and Sato (40).

Superoxide generation was determined by assaying the superoxide dismutase-sensitive production of stable nitroxyl radicals from the reaction of 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine with $O_2^{\cdot-}$ as described in an accompanying report (37). Reactions were carried out in aqueous solutions containing 0.1 M potassium phosphate buffer, pH 7.4, 1.5 mM of the hydroxylamine, and about 0.25 mg microsomal protein in a final volume of 1 ml, and were initiated by the addition of either NADPH or NADH to a final concentration of 1.2 mM. ESR conditions and instrumental settings were the same as described previously (37). Kinetics of the reaction were followed by measuring the increase in intensity of the second line of the three-line nitroxyl radical spectrum as a function of time.

Production of ·OH was determined by assaying the formation of the POBN-HER adduct as described in the accompanying manuscript (37). Reactions were carried out in 0.1 M potassium phosphate buffer, pH 7.4, 0.05 mM ferric EDTA (1:2 chelate), 30 mM POBN, 100 mM ethanol, 0.05 mM sodium azide, about 0.25 mg microsomal protein in a final volume of 1 ml, and were initiated by the addition of either NADPH or NADH to a final concentration of 1.2 mM. Kinetics of the reaction were followed by measuring the intensity of the maximum of the third low field line of the POBN–HER adduct. All ESR measurements were carried out at room temperature in a flat quartz cuvette, using a Bruker E-300 spectrometer, and spectra were recorded about 20 s after initiating the reaction with cofactor.

The phosphate buffer and the water used to prepare solutions were passed through Chelex-100 resin. 4-POBN was purchased from Sigma Chem. Co. (St. Louis, MO) and used without additional purification.

Statistical evaluation was calculated by the two-tailed paired t test, comparing values for microsomes from ethanol-fed rats to those of the pair-fed controls. Values refer to means ± SE.

RESULTS AND DISCUSSION

Chronic ethanol treatment of rats resulted in an increase in the content of cytochrome P450 (0.49 ± 0.03 nmol/mg protein for controls, 0.85 ± 0.07 nmol/mg protein for ethanol treated, +74%) and cytochrome b5 (0.29 and 0.41 nmol/mg protein for controls and for ethanol-treated, respectively, +40%). There were no significant

changes in the activities of NADPH cytochrome P450 reductase or of NADH-cytochrome b5 reductase. The oxidation of ethanol by the microsomes was elevated more than fourfold when expressed on a per milligram protein basis (control rate of nmol acetaldehyde produced per minute per milligram of 4.6 ± 0.6 versus a rate of 20.3 ± 2.5 after ethanol treatment), or nearly threefold when expressed on a per nanomole total P450 basis (control rate of 8.3 ± 1 nmol per minute per nanomole P450 versus a rate of 22 ± 2.6 after the ethanol treatment).

Figure 1a shows the typical three line (triplet) spectrum of the nitroxyl radical produced from the interaction of 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine with superoxide radical. The splitting constants for the resulting triplet were A_N = 16.0 G, g = 2.005. Microsomes from pair-fed controls and from rats chronically fed ethanol when incubated with NADPH display the spectra shown in Figs. 1b and 1c; the intensity of the signal was greater with microsomes after ethanol treatment. NADH also supported production of superoxide with both microsomal preparations, with an elevated signal intensity again being observed with the microsomes from the ethanol-treated rats (Fig. 1e) compared to the pair-fed controls (Fig. 1d). The addition of superoxide dismutase produced more than an 80% decrease in the signal intensity under all conditions, validating that the observed spectra were due to superoxide production.

The kinetics of the reaction were determined by following the intensity of the second line of the triplet with time. Intensity of the signal with both cofactors and with both microsomal preparations were linear over the 10-min incubation period (Fig. 2). The elevated rates of superoxide production after ethanol treatment with either NADPH or NADH as the microsomal reductant is observed at all time points studied. A summary of the results with six pairs of rats is shown in Table I. An elevated rate of superoxide production by microsomes was found with both NADPH and NADH in all the pairs after the ethanol treatment; the extent of increase with NADPH as cofactor was about 50%, while the increase with NADH was about 30%. Rates of superoxide production were about three- to fourfold greater with NADPH than with NADH in both microsomal preparations, similar to results with microsomes from chow-fed control rats (37).

As described in an accompanying report (37) the production of $^{\cdot}OH$ was assayed by measuring the intensity

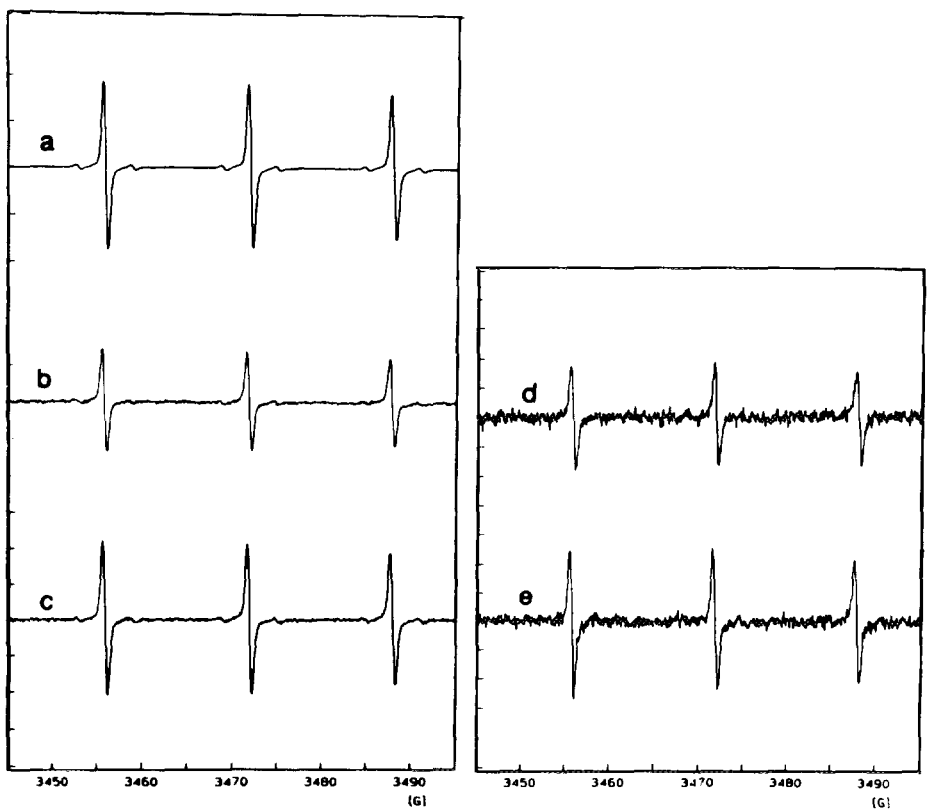

FIG. 1. Detection of superoxide radical from its interaction with 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine. Experiments were carried out as described under Materials and Methods. (a) spectra of a 0.04 mM solution of the standard nitroxyl radical; (b) pair-fed control microsomes plus NADPH; (c) ethanol-treated microsomes plus NADPH; (d) pair-fed controls plus NADH; (e) ethanol-treated plus NADH. Spectra were obtained after a 10-min incubation period. The scale for curves d and e is four times smaller than that for curves b and c, while the scale for curve a is four times larger than that for curves b and c.

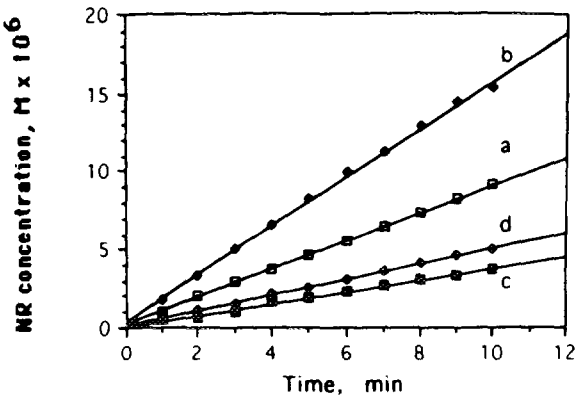

FIG. 2. Kinetics of the production of $O_2^{\cdot}$ by microsomes as a function of incubation time. (a) pair-fed control microsomes plus NADPH; (b) ethanol-treated microsomes plus NADPH; (c) pair-fed controls plus NADH; (d) ethanol-treated plus NADH. NR refers to the nitroxyl radical.

of the POBN-HER adduct. Results in Fig. 3 show the six-line spectra obtained with microsomes from pair-fed control rats when NADPH (Fig. 3a) or NADH (Fig. 3c) were cofactors, while the spectra obtained with microsomes from the ethanol-treated rats incubated with NADPH or NADH is shown in Figs. 3b and 3d, respectively. In all cases, the splitting constants (A_N = 15.7, A_H = 2.63) were identical. Signal intensity was greater with the microsomes from the ethanol-treated rats with both cofactors. The kinetics of the reaction are shown in Fig. 4; reactions were linear for at least 10 min under all conditions. A clear increase in $^{\cdot}OH$ production by microsomes after ethanol treatment is apparent with either NADPH or NADH as cofactor.

A summary of the results with six pairs of rats is shown in Table II. Chronic treatment with ethanol resulted in an increase in microsomal $^{\cdot}OH$ production in all pairs, with either NADPH or NADH as the cofactor. The extent of increase with NADPH was 74%, and that with NADH was 52%. NADH was as effective as NADPH in supporting $^{\cdot}OH$ production with both microsomal preparations. For comparative purposes, previous results obtained with chemical scavengers to detect the production of $^{\cdot}OH$ by microsomes (25, 26, 31) are also shown in Table II. The production of ethylene gas from 2-keto-4-thiomethylbutyric acid (KMB) or of formaldehyde from DMSO was used in these experiments to detect the presence of $^{\cdot}OH$-like species. With NADPH as the reductant, oxidation of KMB and DMSO were elevated 82 and 118% after ethanol treatment, while the POBN-HER adduct was increased 74%. With NADH as the reductant, the increase in oxidation of the chemical scavengers (41 and 50%) after ethanol treatment is identical to the increase found for production of the POBN-HER adduct (52%).

For the above experiments, ferric EDTA was used as the iron catalyst since rates of $^{\cdot}OH$ production by microsomes are very high with this complex (17). When ferric EDTA was omitted and desferrioxamine added to chelate any contaminating iron or iron present in the microsomes (41, 42), the signal intensity was markedly suppressed with both microsomal preparations (Fig. 5). This POBN-HER adduct signal in the presence of desferrioxamine may represent the α-hydroxyethyl radical produced from the direct, radical-independent oxidation of ethanol by cytochrome P450, as described by Albano *et al.* (32, 33) and Reinke *et al.* (34, 35) or a desferrioxamine-resistant, radical-dependent oxidation of ethanol (43, 44). Under our reaction conditions, the desferrioxamine-insensitive POBN-HER adduct could be directly observed without the need for extraction with organic solvents. The intensity of the signal was increased nearly threefold after ethanol treatment (6.5 ± 0.9 mm per milligram protein for pair-fed controls, 16.7 ± 0.3 mm per milligram protein for microsomes from ethanol-fed rats, n = 3), confirming the results of Albano *et al.* (32, 33) and Reinke *et al.* (34, 35). This increase most likely reflects the induction of cytochrome P4502E1 after the ethanol treatment (45).

Microsomes from both preparations displayed similar effects by anti-oxidants (Fig. 6). Catalase completely prevented production of the POBN-HER adduct, indicating the requirement for H_2O_2. As found with microsomes from chow-fed controls, superoxide dismutase had no effect on formation of the adduct. Competing scavengers such as mannitol and GSH decreased intensity of the POBN-HER signal.

These results indicate that production by microsomes of reactive oxygen intermediates such as superoxide radical and $^{\cdot}OH$ is increased after chronic ethanol treatment as determined by ESR spectroscopic analysis. Two previous reports have shown increases in superoxide radical

TABLE I

Effect of Chronic Ethanol Treatment on Microsomal Production of Superoxide Radical

	Superoxide production—NADPH		Superoxide production—NADH	
Pair	Control	Ethanol	Control	Ethanol
1	3.51	5.26 (50)	0.89	1.50 (69)
2	3.34	4.54 (36)	0.90	1.21 (34)
3	3.62	4.49 (24)	0.96	1.22 (27)
4	3.91	5.49 (40)	1.47	1.72 (17)
5	3.30	5.70 (73)	1.00	1.30 (30)
6	3.41	5.80 (70)	0.97	1.33 (39)
n = 6	3.51 ± 0.23	5.21 ± 0.57 (48)	1.03 ± 0.09	1.38 ± 0.03 (34)
		$P < 0.001$		$P < 0.001$

Note. Superoxide radical production was determined as described under Materials and Methods, in the presence of either NADPH or NADH. Rates refer to nmol per minute per milligram protein and were determined by comparing signal intensities to that of the nitroxyl radical standard. Numbers in parentheses refer to the percentage stimulation by the ethanol treatment.

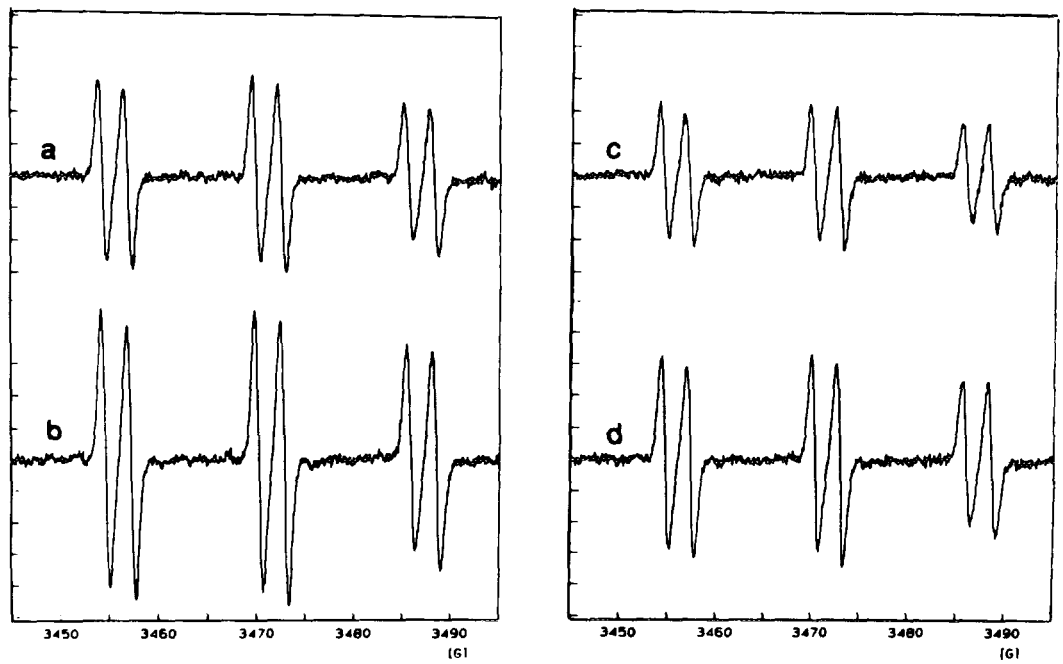

FIG. 3. Formation of POBN–HER adducts by microsomes. Reactions were carried out as described under Materials and Methods and spectra were recorded after a 15-min incubation period. (a) pair-fed controls plus NADPH; (b) ethanol-treated plus NADPH; (c) pair-fed controls plus NADH; (d) ethanol-treated plus NADH.

production after ethanol treatment; in one (18), a non-specific, autocatalytic epinephrine oxidation analytical method was used, while in the other (19), a more specific reduction of succinylated cytochrome C was the analytic method employed. NADPH was the reductant in both previous studies. Ekstrom and Ingelman-Sundberg (19) reported control rates of superoxide production of 2 nmol/min/mg protein and rates of 5.2 nmol/min/mg after ethanol treatment, compared to NADPH-dependent rates of 3.5 and 5.2 nmol/min/mg as detected by ESR spectroscopy. The extent of increase in superoxide production after ethanol treatment in their study (160%) is greater than that observed in our study (48%), which could be due to the much higher total content of cytochrome P450 in their preparations (0.65 nmol/mg for controls, 1.65 nmol/mg for ethanol-treated, +158% as compared to an increase of 74% in our preparations). Indeed in both studies, the increase in superoxide production appears to

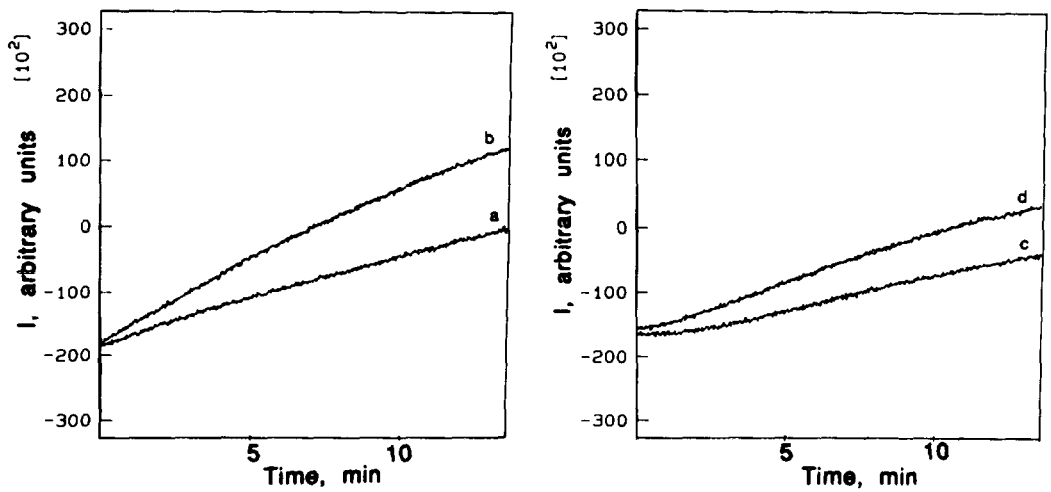

FIG. 4. Time course for the production of the POBN–HER adduct. (a) pair-fed controls plus NADPH; (b) ethanol-treated plus NADPH; (c) pair-fed controls plus NADH; (d) ethanol-treated plus NADH.

TABLE II

Effect of Chronic Ethanol Treatment on Microsomal Production of Hydroxyl Radical

	POBN-HER adduct—NADPH		POBN-HER adduct—NADH	
Pair	Control	Ethanol	Control	Ethanol
A. 1	6.5	9.3 (43)	7.3	10.0 (36)
2	7.2	12.9 (80)	7.4	10.9 (48)
3	8.0	14.8 (85)	6.1	9.2 (52)
4	6.5	10.3 (59)	6.2	9.4 (52)
5	6.5	9.9 (52)	6.9	12.0 (75)
6	6.2	13.5 (117)	9.3	14.3 (54)
$n = 6$	6.8 ± 0.27	11.8 ± 0.9 (74)	7.2 ± 0.48	11.0 ± 0.81 (52)
		$P < 0.001$		$P < 0.001$
B. KMB	9.49 ± 0.34	17.31 ± 1.53 (82)	6.21 ± 0.42	8.78 ± 0.5 (41)
DMSO	4.37 ± 0.41	9.53 ± 2.13 (118)	3.30 ± 0.40	4.95 ± 0.9 (50)

Note. Hydroxyl radical production was determined by measuring intensity of the spectra of the POBN-HER adduct after 15 min of reaction. Values refer to mm per minute per milligram protein of the third line, peak to trough, of the spectra. Numbers in parentheses refer to effect of the ethanol treatment. Results for experiment B have been previously published (25, 26, 31) and are intended to allow comparisons of the ESR results with those previously obtained with chemical scavengers—ethylene production from 2-keto-4-thiomethylbutyric acid and formaldehyde production from DMSO. These results refer to nmol per minute per milligram protein.

correlate closely with the increase in total content of cytochrome P450. NADH is also capable of supporting microsomal superoxide production, although at a lower rate than the NADPH-dependent reaction. Microsomes from the ethanol-treated rats also display elevated production of superoxide with NADH as the cofactor, and the 34% increase in superoxide production correlates with the increase in cytochrome b5 concentration (40%). The role of P450, and especially P4502E1 in the NADH-dependent increase will require studies with inhibitors and antibodies specific for this isozyme.

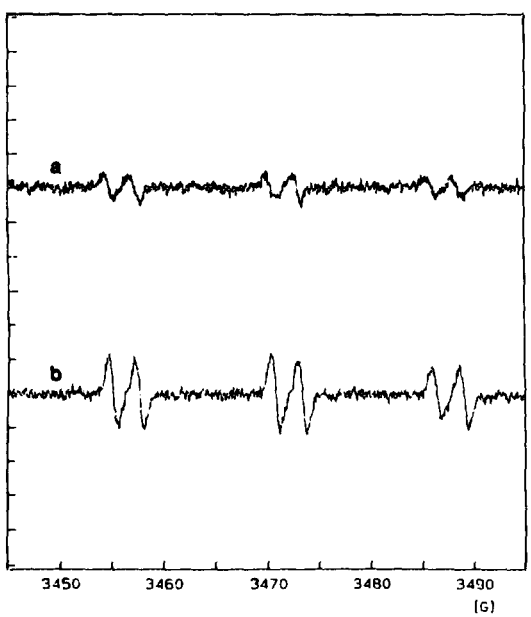

FIG. 5. Formation of the POBN-HER adduct in the presence of desferrioxamine. Reactions were carried out as described under Materials and Methods except for the omission of ferric EDTA and the addition of 100 μM desferrioxamine. (a) pair-fed controls plus NADPH; (b) ethanol-treated plus NADPH.

Chronic ethanol treatment increases microsomal generation of H_2O_2 (20, 21). We had previously reported increases in H_2O_2 production of 120 and 64% with NADPH and NADH, respectively (31), which is somewhat greater than the increases in superoxide production. Rates (nmol/min/mg protein) of NADPH-dependent production of H_2O_2 (6.6 for controls, 14.5 for ethanol-treated) are about twice those for superoxide production (3.5 for controls, 5.2 for ethanol-treated, Table I). In a similar manner, rates of NADH-dependent production of H_2O_2 (1.4 for controls, 2.3 for ethanol-treated) are also about twice those for superoxide production (0.9 for controls, 1.5 for ethanol-treated, Table I). If superoxide dismutation was the only source of H_2O_2, production of H_2O_2 would be about half that of superoxide production. The higher rates of H_2O_2, compared to superoxide generation by both microsomal preparations imply that a considerable amount of the H_2O_2 produced is derived from sources independent of dismutation of superoxide radical, with either NADPH or NADH as reductant, or may be due to the different analytical procedures utilized to detect the two oxidizing species.

Previous results using chemical scavengers such as KMB or DMSO suggested that microsomes generate ·OH-like species at elevated rates after ethanol treatment (17, 25–28). Since chemical scavengers such as KMB can react with other oxidants besides ·OH to yield ethylene (46), spin-trapping studies were carried out to assess in a more specific and direct manner the production of ·OH. Production of the POBN-HER adduct by microsomes was

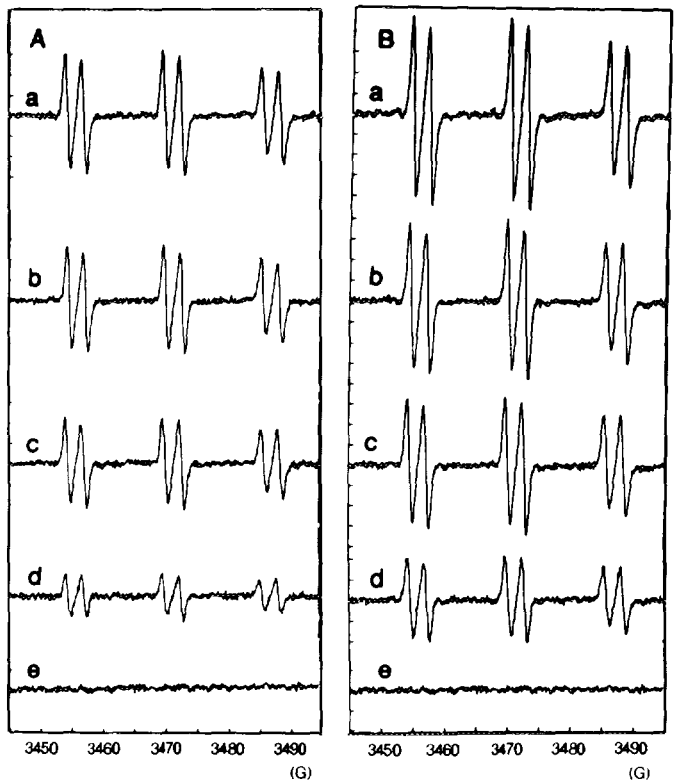

FIG. 6. Effect of anti-oxidants on formation of the POBN-HER adduct by pair-fed control microsomes (A) or by microsomes from ethanol-treated rats (B). (a) control incubations; (b) 25 munits/ml superoxide dismutase; (c) 10 mM GSH; (d) 200 mM mannitol; (e) 25 munits/ml catalase.

increased after ethanol treatment with either NADPH or NADH. The extent of increase as detected by ESR spectroscopy is similar to the increase previously found with assays of oxidation of chemical scavengers. The extent of increase in ·OH production (74% NADPH, 52% NADH) is similar to the extent of increases of superoxide and H_2O_2 production by the microsomes after ethanol treatment. In view of the inhibition of ·OH production by catalase and the lack of effect of superoxide dismutase, increases in H_2O_2 production probably play an important role in the increased production of ·OH after ethanol treatment.

In summary, we provide evidence from ESR spectroscopic determination of adducts of spin-trapped radicals that microsomes from ethanol-fed rats are more reactive in generating superoxide and ·OH than pair-fed controls. Increased production of reactive oxygen intermediates by microsomes may contribute to the hepatotoxic actions of ethanol. Increases in oxygen radical production are found with both NADPH and NADH as the cofactors. Although the extent of increase is greater with NADPH, oxidation of ethanol by alcohol dehydrogenase produces NADH in the liver, and this elevated production of NADH may also contribute to increases in reactive oxygen generation by microsomes after ethanol treatment.

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