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ESR Studies on the Production of Reactive Oxygen Intermediates by Rat Liver Microsomes in the Presence of NADPH or NADH

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Experiments were carried out using spin-trapping ESR spectroscopy to evaluate in a quantitative and kinetic manner the production of reactive oxygen intermediates by rat liver microsomes. Comparisons between the effectiveness of NADH and that of NADPH in catalyzing microsomal generation of reactive oxygen intermediates were made. Superoxide production was determined by assaying the generation of superoxide dismutase-sensitive stable nitroxyl radicals formed from 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine. Identical spectra were produced when microsomes were incubated with NADH or NADPH; reaction rates were linear for at least 10 min of reaction and were about three- to fourfold greater with NADPH. In the presence of iron, microsomes catalyze the production of hydroxyl radical during electron transfer. Initial experiments utilizing 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) as the spin-trapping agent proved unsatisfactory for the microsomal system as adequate kinetics could not be determined in view of the rapid decay of the DMPO–OH adduct, as well as secondary adducts such as DMPO–CH_3 or DMPO–hydroxyethyl (HER). The spin-trapping agent α-[4-pyridyl-1-oxide]-*N-tert*-butylnitrone (POBN) was evaluated. POBN–OH adducts were somewhat more stable but also rapidly decayed after 2 to 3 min. However, production of POBN–CH_3 and POBN–HER adducts was proportional to that of microsomal protein, increased with time over a 5- to 15-min period, and then was relatively stable. The redox cycling agent paraquat increased POBN–HER adduct formation twofold. Formation of the adduct with either NADH or NADPH required an iron catalyst and did not occur in the presence of the iron chelator desferrioxamine. Ferric EDTA was most reactive in catalyzing production of the adduct, ferric DTPA was 60 to 70% as effective, and ferric ATP was 20 to 30% as effective as ferric EDTA, with both reductants. Irrespective of the iron complex, rates of POBN–HER formation with NADH were about 70% those of NADPH. Formation of the adduct was inhibited by catalase, mannitol, and GSH, but not superoxide dismutase. These experiments support the usefulness of POBN plus ethanol for kinetic studies on the production of ·OH by microsomes and validate that microsomes in the presence of an iron catalyst generate ·OH not only with NADPH as cofactor, but also with NADH.

Isolated microsomes produce superoxide radical ($O_2^{\cdot-}$)[2] and H_2O_2 during NADPH-dependent electron transfer (1–8). Most of these reactive oxygen intermediates are believed to arise from the decay of oxygenated cytochrome P450 intermediates in the overall pathway of mixed-function oxidation (5, 6, 8, 9). In the presence of iron, superoxide and H_2O_2 may serve as precursors for the production of hydroxyl radical (·OH)-like species produced by Fenton or Haber–Weiss types of reactions (10–12). The role of iron in catalyzing microsomal production of reactive oxygen intermediates is complex and dependent on the nature of the chelated form of the iron. Certain complexes such as ferric EDTA and ferric DTPA are very effective in generating ·OH-like species, while ferric ADP, ferric ATP, or ferric citrate are much less effective. However, the latter three ferric complexes are much more effective than ferric EDTA or ferric DTPA in catalyzing microsomal lipid peroxidation and light emission (13–17). Many of these studies have utilized a variety of chemical

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[2] Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; POBN, α-[4-pyridyl-1-oxide]-*N-tert*-butylnitrone; ·OH, hydroxyl radical, or a species with the oxidizing power of the hydroxyl radical; $O_2^{\cdot-}$, superoxide anion radical; HER, hydroxyethyl radical; DMSO, dimethyl sulfoxide; Tempo, 2,2,6,6-tetramethylpiperidinooxy, free radical.

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techniques to evaluate the production of ·OH-like species by microsomes, including oxidation of alcohols to aldehydes, production of ethylene from methional and 2-keto-4-thiomethylbutyric acid, and the production of formaldehyde from DMSO or *t*-butyl alcohol (18). However, alcohols can be oxidized by direct cytochrome P450-catalyzed pathways independent of oxygen radicals (19–21) and scavengers such as ethanol, methional, or 2-keto-4-thiomethylbutyric are not specific for ·OH but react with other oxidants, e.g., alkoxyl and peroxyl radicals, to yield acetaldehyde or ethylene (22, 23). More specific and direct detection techniques such as ESR spectroscopy would be helpful in validating the production of ·OH by microsomes in the presence of different ferric complexes and is a goal of the current report.

Most studies on the generation of reactive oxygen intermediates by microsomes have utilized NADPH as the cofactor since this is an effective reductant for the mixed-function oxidase system. The interaction of NADH with microsomes and with various ferric complexes to catalyze microsomal generation of reactive oxygen intermediates has not been as well studied as the NADPH-dependent reactions. NADH-dependent rates of microsomal lipid peroxidation were about 10 to 30% of the NADPH-dependent rates when ferric ADP was the iron catalyst (24–29). Purified NADH-cytochrome b5 reductase reduces ferric EDTA, and in the presence of ferric EDTA plus ferric ADP, rates of NADH- and NADPH-dependent lipid peroxidation were equivalent (28). In the presence of iron, NADH catalyzed the oxidation of chemical scavengers at rates which were about 50 to 80% the NADPH-dependent rates, and ferric EDTA was much more effective than ferric ATP in catalyzing NADH-dependent microsomal generation of ·OH-like species (30). There do not appear to be any reports in the literature using ESR spectroscopy to specifically assay NADH-dependent production of ·OH by microsomes and comparing these rates with those found with NADPH.

Spin-trapping ESR has been used to demonstrate microsomal production of radicals associated with the metabolism of CCl_4 and other halogenated compounds, anesthetic agents such as halothane, hydrazines, alcohols, and radicals produced during the lipid peroxidation cascade [reviewed in Ref. (31)]. Piette and co-workers (32, 33) were among the first to use ESR spin-trapping to demonstrate the production of ·OH by microsomes and by NADPH–cytochrome P450 reductase. 5,5-Dimethyl-1-pyrroline 1-oxide (DMPO) was used as the spin-trapping agent, ferric EDTA as the iron catalyst, and NADPH as the reductant (32, 33). Recently, Yamazaki *et al.* (34) used DMPO to show that the reductase in the presence of paraquat produced superoxide, while ·OH was produced when ferric EDTA and H_2O_2 were added. A variety of redox cycling quinones produced DMPO–OH adducts in the presence of ferric EDTA plus either microsomes or the reductase, and NADPH was more effective than NADH (35). Cobalt (36) or chromium (37) could replace iron in catalyzing NADPH-dependent production of the DMPO–OH adduct by rat liver microsomes. Several problems associated with the use of DMPO as a spin-trapping agent such as decay of a DMPO–OOH adduct to a DMPO–OH signal, instability, and bioreduction of the DMPO–OH adduct in biological systems, and decay of DMPO–OH in the presence of superoxide have been reported (38–41). Ramos *et al.* (42) and Pou *et al.*[3] have recently reported that α-[4-pyridyl-1-oxide]-*N*-*tert*-butylnitrone (4-POBN) may be preferable to DMPO, especially when used with ethanol, as the α-hydroxyethyl radical (HER) adduct of POBN is stable, especially in the presence of superoxide, and HER has a greater rate of reaction with POBN than with DMPO or PBN. Gonthier *et al.* (43) recently used POBN to detect the production of ·OH generated by liver and brain microsomes in the presence of NADPH and ferric DTPA. The goal of the present work was to compare NADPH and NADH as cofactors for microsomal production of reactive oxygen intermediates and to compare the effects of several ferric complexes on ·OH generation by a direct ESR spin-trapping method. An accompanying paper (44) is concerned with the effects of chronic ethanol consumption on microsomal production of superoxide and ·OH as detected by ESR. Initial experiments were carried out to optimize experimental conditions with either DMPO or POBN in order to have a more quantitative method for comparison, based on kinetics rather than just spectra.

MATERIALS AND METHODS

Liver microsomes were isolated from male Sprague–Dawley rats weighing about 200 g. The rats were starved overnight prior to being killed. Animals were anesthesized with pentobarbital (150 mg/kg) and killed by decapitation. Liver homogenates (1:10) were prepared and microsomes were isolated by differential centrifugation, washed two times, resuspended in 0.125 M KCl, 0.01 M potassium phosphate, pH 7.4, and stored at −70°C at a protein concentration of about 20 mg/ml. Mitochondrial contamination in microsomes, as determined by measuring the enzyme marker succinate dehydrogenase was less than 3%. Protein was determined by the method of Lowry *et al.* (45).

Superoxide generation was determined by assaying the production of stable nitroxyl radicals produced from the reaction of hydroxylamines with $O_2^{\cdot-}$ (40). The hydroxylamine, 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine, was synthesized (46, 47) and dissolved in triply distilled, Chelex-treated water. Rat liver microsomes (usually 0.3 mg per milliliter) were incubated in 100 mM potassium phosphate buffer, pH 7.4, plus 1.5 mM of the hydroxylamine at room temperature and reactions were initiated by the addition of either NADPH or NADH to a final concentration of 1.2 mM. The samples were immediately transferred to a WG-812Q flat quartz cuvette (EPR Wilmad Co.) and spectra recorded with a Bruker E-300 spectrometer, Probe 4102ST. Kinetics of the reaction were followed as a function of time by measuring the increase in intensity of the second line of the three-line nitroxide radical spectrum. A standard spectrum was carried out with the stable nitroxyl radical, 4-oxo-Tempo

[3] Pou, S., Ramos, C. L., Renks, E., Centra, M., Young, D., Kang, G., Cohen, M. S., and Rosen, G. M. Personal communication. We thank Dr. Rosen for providing a preprint of this work to us.

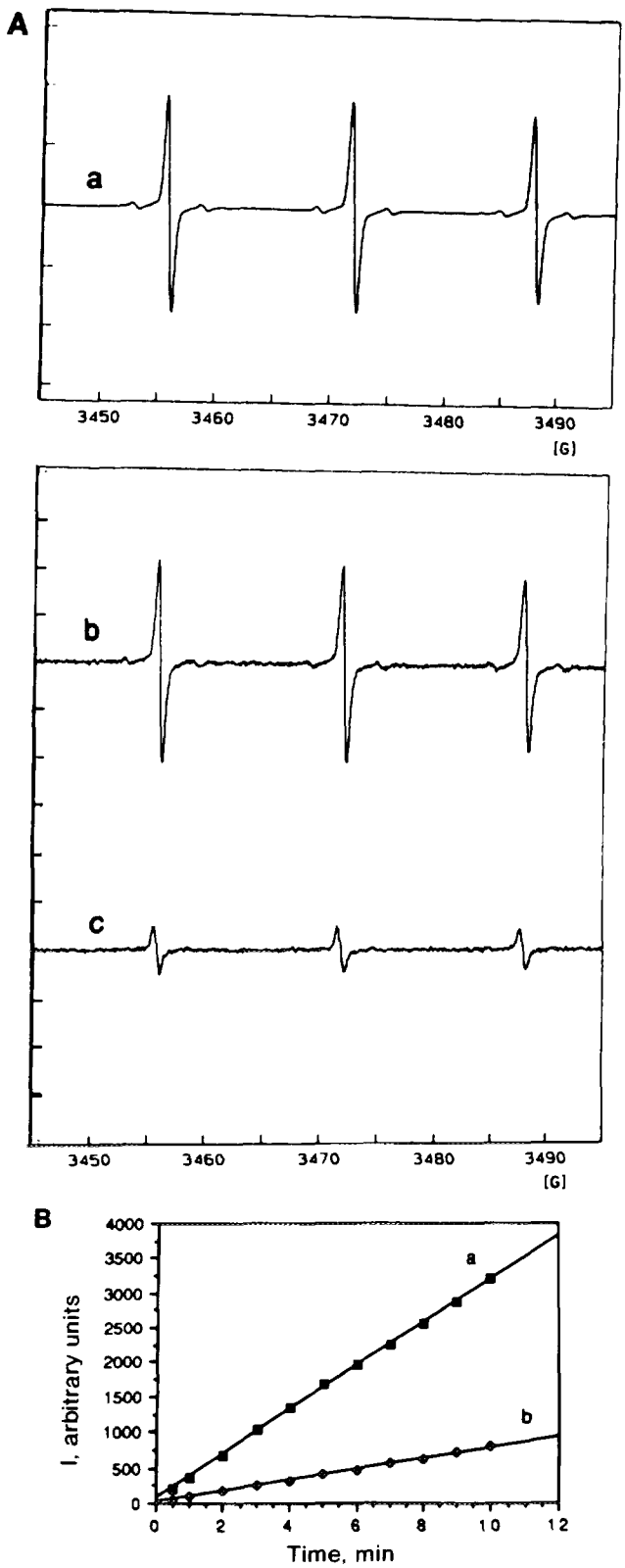

FIG. 1. Detection of superoxide via interaction with 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine. Experiments were carried out as described under Materials and Methods. A: (a) Spectra of a 0.04 mM aqueous solution of the standard nitroxyl radical; (b) spectra after a 10-min incubation of microsomes with NADPH; (c) spectra after a 10-min incubation of microsomes with NADH. B: Kinetics of the intensity of the ESR signal versus incubation time. (a) NADPH and (b) NADH as the cofactor.

(Aldrich Chem. Co., Milwaukee, WI). Instrument settings were as follows: for spectra; sweep width 70.0 G, sweep time 20.97 s, time constant 10.24 ms, modulation amplitude 0.5 G, modulation frequency 100 kHz, microwave power 11 mW, temperature 20°C.

A variety of systems were utilized to generate $^{\cdot}OH$. Photolysis of H_2O_2 was carried out using 30 to 100 mM H_2O_2 in water and the full light of a 1000-W Xe–Hg ORIEL lamp with irradiation for 1 to 3 min. The H_2O_2 solution was irradiated directly in the cavity. The Fenton system consisted of 0.2 to 0.4 mM H_2O_2 plus 0.1 mM ferrous ammonium sulfate. The xanthine oxidase system consisted of 0.1 M phosphate buffer, pH 7.4, 0.14 mM hypoxanthine, 0.05–0.10 mM ferric EDTA (1:2 chelate) plus 0.05 units of xanthine oxidase to initiate the reaction, in a final volume of 0.5 ml. The xanthine oxidase was passed through a Sephadex G-25 column prior to use to remove low molecular weight stabilizers and anti-oxidants. This amount of xanthine oxidase resulted in rates of superoxide production of 5 to 20 μM per minute as determined by assaying for SOD-inhibitable rates of reduction of cytochrome C as measured by increased absorbance at 550 nm. The microsomal system contained 0.1 M phosphate buffer, pH 7.4, 0.05 mM sodium azide, microsomes (usually 0.3 mg protein per milliliter), 0.05 mM ferric EDTA (or other ferric complexes as described), and either NADPH or NADH (1.2 mM final concentrations) to initiate the reaction. When present, final concentrations of ethanol or DMSO were 100 mM. Two spin-trapping agents were used; DMPO, at a final concentration of 70 mM, and POBN, at a final concentration of 30 mM. ESR measurements were started about 20 to 30 s after initiation of the reaction, and were carried out at room temperature typically over a 15-min time period. Kinetics for formation of the POBN adducts were carried out by measuring the intensity of the maximum of the third low field line of the spin-trapped adduct. Instrument settings were as above, except that for kinetics the following changes were made: modulation amplitude 0.2 G, sweep width 0 G, and sweep time 15 min.

The spin traps 4-POBN and DMPO were purchased from Sigma Chem. Co. (St. Louis, MO). DMPO was further purified by passing through charcoal (48). POBN was used without additional purification. Superoxide dismutase, catalase, and xanthine oxidase were from Boehringer Mannheim (Indianapolis, IN). Most other chemicals were from Sigma. The phosphate buffer and the water were passed through Chelex-100 (Bio-Rad, Richmond, CA) resin to remove metal contaminants. The ferric complexes were prepared by dissolving ferric ammonium sulfate in 0.1 N HCl and then diluting with the respective chelating agent to the appropriate stock concentration. Ferric EDTA and ferric DTPA were used as 1:2 iron:chelator complexes, while ferric ATP was used as a 1:20 iron:ATP complex.

RESULTS

Superoxide production by microsomes. Hydroxylamines react with $O_2^{\cdot-}$ to produce a stable nitroxyl radical which can readily be detected by ESR (40). 1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine has a rate constant of 2.1×10^3 M^{-1} s^{-1} for interacting with $O_2^{\cdot-}$ and has been used to detect production of $O_2^{\cdot-}$ by the xanthine oxidase reaction and by rat liver nuclei and submitochondrial particles (46, 47). With submitochondrial particles, NADH-dependent production of $O_2^{\cdot-}$ was fourfold greater than NADPH-dependent rates of $O_2^{\cdot-}$ generation (46). Figure 1a shows the spectrum of the Tempo nitroxide

standard; the resulting triplet displayed the following constants; A_N = 16.0 G and g = 2.0050. Well-washed microsomes were incubated with the hydroxylamine and either NADPH or NADH; the spectra obtained after a 10-min incubation are shown in Figs. 1b and 1c, respectively. By following the intensity of the second line of the triplet, rates of O_2^- production were linear for at least 10 min of reaction with both cofactors (Fig. 1B). Rates of O_2^- production were (nmol/min/mg protein) 3.2 with NADPH and 0.8 with NADH as the reductant. Superoxide dismutase (25 units per milliliter) produced greater than 80% inhibition of the spectrum found in the presence of either NADPH or NADH (data not shown).

Use of DMPO as a spin-trapping agent. Initial studies utilized the photolysis of H_2O_2 by uv irradiation to generate $^{\cdot}OH$; the characteristic four-line spectra of DMPO–OH was readily detected with splitting constants of A_N = 15.0 G and A_H = 15.0 G, identical to values reported in the literature (32, 33, 38, 42). The resulting DMPO–OH adduct was very stable with little or no decay in these chemical or photolysis systems. However, DMPO proved to be unsuitable as a spin-trapping agent for detection of $^{\cdot}OH$ in microsomal systems as we could not detect a definitive DMPO–OH ESR signal. In view of concerns of the decay of the DMPO–superoxide adduct (DMPO–OOH) to a DMPO–OH adduct, several investigators have scavenged the primary $^{\cdot}OH$ radical with $^{\cdot}OH$ scavengers to produce a secondary radical and detected the secondary radical–DMPO adduct to validate the presence of $^{\cdot}OH$ in the reaction system (31, 42). Ethanol and DMSO have been widely utilized for this purpose (31, 42, 49, 50). With rat liver microsomes incubated with NADPH, ferric EDTA, and either ethanol or DMSO, secondary radical signals identical to those of literature reports (31, 42, 49, 50) were observed; however, these signals again proved to be too unstable for kinetic and quantitative analysis as maximal signal intensity was reached within a minute, followed by rapid decay. Despite efforts to inhibit cytochrome P450 or NADPH cytochrome P450 reductase (metyrapone, *p*-chloromercuribenzoate) or remove O_2^- or H_2O_2 (SOD, catalase) after the signal maximum, we could not prevent rapid decay of the DMPO–OH adduct by microsomes, confirming the results of Rosen and co-workers (38–42). We therefore evaluated the effectiveness of POBN as a spin-trapping agent in microsomal systems.

POBN as a spin-trapping agent. Figure 2 shows results of $^{\cdot}OH$ generated by a Fenton system in the presence of POBN (2a), POBN plus ethanol (2b), and POBN plus DMSO (2c). Splitting constants for the POBN–OH adduct were A_N = 15.1 G and A_H = 1.63 G, and for the POBN–HER adduct, A_N = 15.67 G and A_H = 2.63 G, in agreement with values in the literature (42, 43, 51–54). With DMSO, two overlapping signals were observed, most likely reflecting POBN–methyl and POBN–methoxy radical adducts. Splitting constants for the POBN–CH_3 adduct were A_N = 15.51 G and A_H = 2.72 G, in agreement with literature results (55); splitting constants for the POBN–OCH_3 adduct were A_N = 14.41 G and A_H = 2.19 G, in agreement with unpublished results of Rosen and co-workers.[3] Anaerobiosis prevented production of the POBN–OCH_3 but not the POBN–CH_3 adduct. With the xanthine oxidase system, essentially similar results were obtained except that with DMSO, only the POBN–CH_3 adduct was detected. With rat liver microsomes, a weak signal of the POBN–OH adduct could be detected, but the signal rapidly decayed and disappeared by 2 to 3 min. However, stable POBN–HER and POBN–CH_3 (we did not detect POBN–OCH_3 adducts in microsomes) signals were detected (Figs. 2d and 2e). Monitoring these secondary radical signals permitted kinetics of the reaction to be followed; as shown in Fig. 3, the POBN–HER and POBN–CH_3 adducts continued to increase over 10- and 5-min reaction periods, respectively. The intensity of the

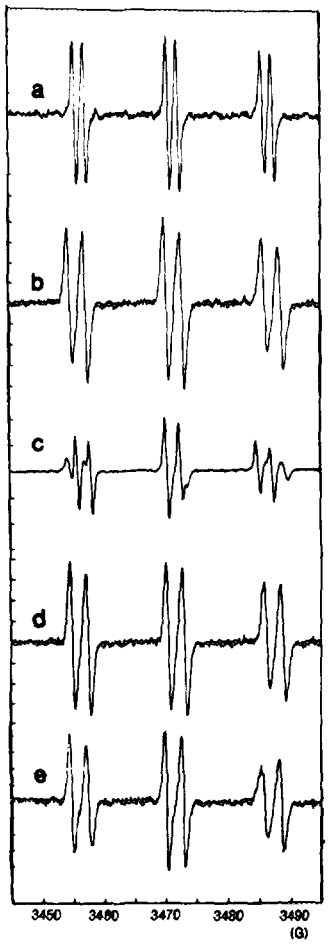

FIG. 2. ESR spectra with POBN. The top three curves refer to signals obtained by a Fenton system in the absence of any further additions (a), in the presence of 100 mM ethanol (b), or in the presence of 100 mM DMSO (c). Curves (d) and (e) refer to experiments with microsomes incubated in the presence of NADPH, POBN, ferric-EDTA, and either ethanol (d) or DMSO (e).

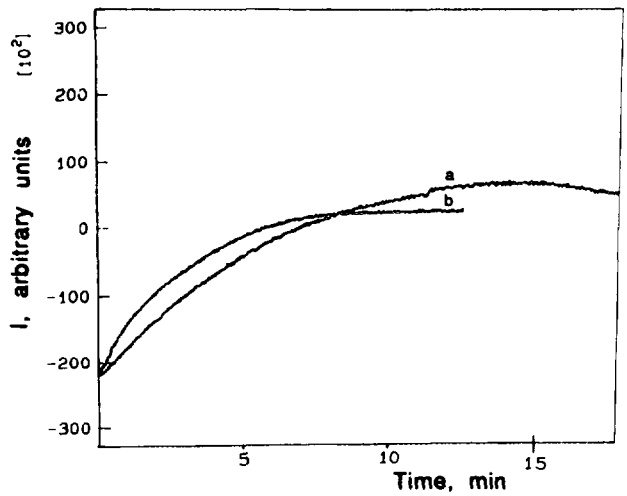

FIG. 3. Time dependence for the production of POBN-HER (a) or POBN-methyl (b) spin-trapped adducts. Experiments were carried out as described under Materials and Methods in the presence of NADPH, POBN, ferric-EDTA, and rat liver microsomes (1.3 mg per milliliter) and either ethanol (a) or DMSO (b).

POBN–HER adduct signal was linear with the amount of microsomal protein, up to about 0.6 mg protein per milliliter (Fig. 4A) and linear time dependent increases in the intensity of the POBN–HER signal was observed at microsomal protein concentrations ranging from 0.1 to 0.5 mg per milliliter (Fig. 4B). Varying the concentration of POBN from 5 to 100 mM indicated that at 0.5 mg microsomal protein per milliliter saturation of the adduct signal was reached at a POBN concentration of 10 mM; for most experiments, POBN was routinely used at a final concentration of 30 mM to ensure efficient trapping of $^{\cdot}OH$.

Effect of paraquat. Paraquat reacts with microsomes in the presence of NADPH to produce a large increase in the production of $O_2^{\cdot-}$ and H_2O_2 as a consequence of its redox cycling ability. In the presence of ferric complexes, paraquat increases production of $^{\cdot}OH$ by microsomes (56, 57) as well as by NADPH–cytochrome P450 reductase (34, 58). Paraquat increased the POBN–HER adduct signal more than twofold compared to that obtained with ferric EDTA alone (Fig. 5A). After a slight temporal lag, the intensity of the POBN–HER adduct signal in the presence of paraquat was linear over a 15-min time period, and at all time points, the increase in $^{\cdot}OH$ production produced by paraquat was readily detected (Fig. 5B).

Comparison of NADPH with NADH. The above results indicated that appropriate conditions could be found using POBN and ethanol to allow adequate comparisons to be made between the effectiveness of varying cofactors and ferric complexes in catalyzing microsomal $^{\cdot}OH$ production. In the presence of ferric EDTA, POBN, and ethanol, identical spectra were observed in the presence of either NADH or NADPH as the microsomal reductant (data not shown) and both cofactors catalyzed a time-dependent production of the POBN–HER adduct (Fig. 6). Rates with NADH were about 70% as high as those obtained with NADPH. With both cofactors, production of the POBN–HER adduct required the presence of a reductant, microsomes, and ferric EDTA (Fig. 7). Replacing EDTA with desferrioxamine as the iron chelating agent resulted in complete loss of the ESR signals; desferrioxamine markedly inhibits the production of $^{\cdot}OH$ by microsomes with either NADPH or NADH as cofactor (16, 17, 30).

Recent results have indicated that even in the presence of desferrioxamine, microsomes can generate ESR detectable HER spin-trapped adducts (54, 59–61). This pathway appears to involve direct oxidation of ethanol by cytochrome P450 by oxygen radical independent pathways. Under conditions in which oxygen radical dependent POBN–HER adduct formation was readily detected (presence of ferric EDTA), an oxygen-radical independent pathway (presence of desferrioxamine) for POBN–HER

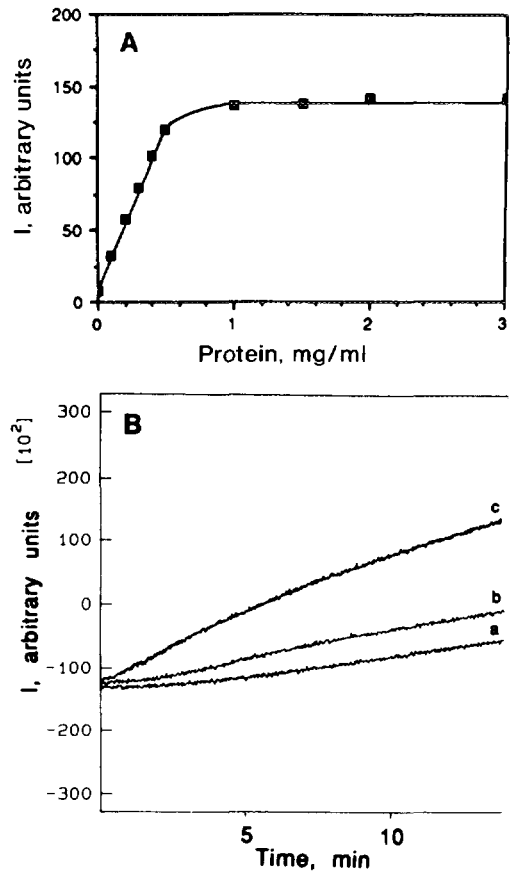

FIG. 4. Microsomal protein concentration curve for the production of the POBN-HER adduct (A). Reaction system consisted of NADPH, ferric-EDTA, POBN, and ethanol and the indicated concentrations of microsomal protein. (B) Time dependence for the production of the POBN-HER adduct is shown at microsomal protein concentrations of 0.1 (a), 0.2 (b), and 0.5 (c) mg per milliliter.

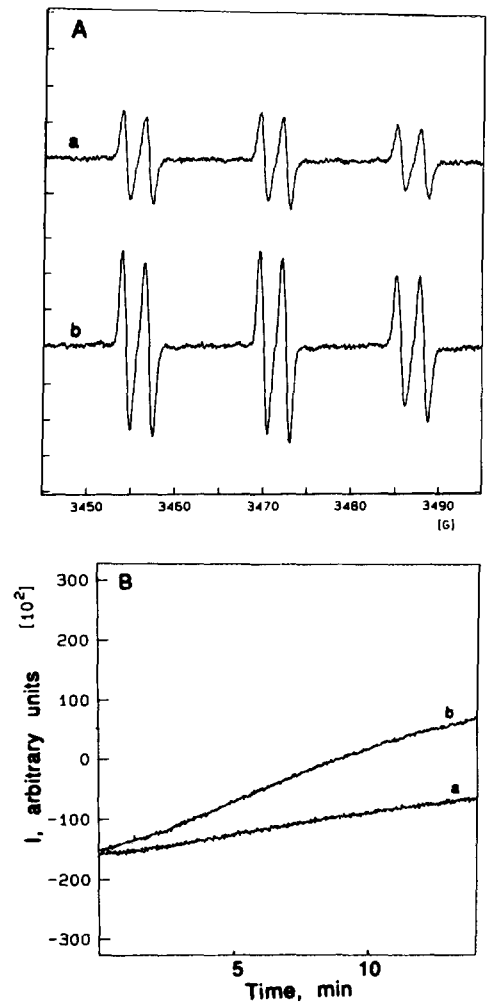

FIG. 5. The effect of paraquat on the production of the POBN-HER adduct. A: ESR spectra experiments were carried out for 15 min with microsomes (0.3 mg per milliliter), NADPH, ferric-EDTA, POBN, and ethanol in the absence (a) and presence (b) of 0.5 mM paraquat. (B) Time dependence for the stimulation of POBN-HER adduct formation by paraquat (b) is shown compared to rates in the absence of paraquat (a).

adduct formation could not be observed. However, when the microsomal protein concentration was elevated fivefold, a weak POBN-HER signal could be directly observed even in the presence of desferrioxamine when NADPH was the microsomal reductant (Fig. 7e).

Effect of ferric chelates. A comparison was made of the effectiveness of ferric EDTA, ferric DTPA, and ferric ATP in catalyzing NADPH- or NADH-dependent production of the POBN-HER adduct. With both reductants, the rank order of effectiveness was ferric EDTA > ferric DTPA > ferric ATP (Fig. 8). Ferric DTPA was about 60 to 70% as effective as ferric EDTA in catalyzing production of the POBN-HER adduct, while ferric ATP was 20 to 30% as effective with either NADPH or NADH. The NADH-dependent rates were about 60 to 70% of those of the NADPH-dependent rates, irrespective of the iron catalyst.

Effect of anti-oxidants. A variety of anti-oxidative agents was tested for their effects on the production of the POBN-HER adduct in order to evaluate the mechanism for $^{\cdot}OH$ production. Superoxide dismutase, at a concentration which strongly inhibits the hydroxylamine–superoxide ESR signal, had no effect on formation of the POBN-HER adduct (Fig. 9b, compared to 9a). Mannitol, added as a competitive $^{\cdot}OH$ scavenger, produced about 60% inhibition when present in twofold excess over the ethanol concentration (Fig. 9d), while GSH produced about 30% inhibition when present at 10% the ethanol concentration (Fig. 9c). Catalase completely eliminated the POBN-HER adduct signal (Fig. 9e).

Since small amounts of catalase can contaminate isolated microsomal preparations, azide was routinely added to the incubation mixture to inhibit catalase. When azide was present at final concentrations of 0.5 to 1 mM, an ESR spectrum consisting of a triplet of quartets, with splitting contants of A_N = 14.7 G, A_H^β = 1.92 G, and A_N^β = 1.92 G, was observed with POBN as spin trap. Similar azidyl adducts with DMPO have been reported (62, 63). In model chemical systems, when the azide concentration was lowered to about 0.05 mM, no POBN-azide spin adduct could be detected in the presence of 100 mM ethanol (POBN-HER spin adduct was detected). Therefore, azide was routinely used at a final concentration of 0.05 mM in microsomal experiments, and at concentrations ranging from 0.05 to 1 mM, the azide produced about a 20 to 30% increase in the POBN-HER adduct signal.

DISCUSSION

During the mixed-function oxidation cycle, isolated microsomes produce superoxide and H_2O_2 (1–9). Superoxide is largely produced from the oxycytochrome P450

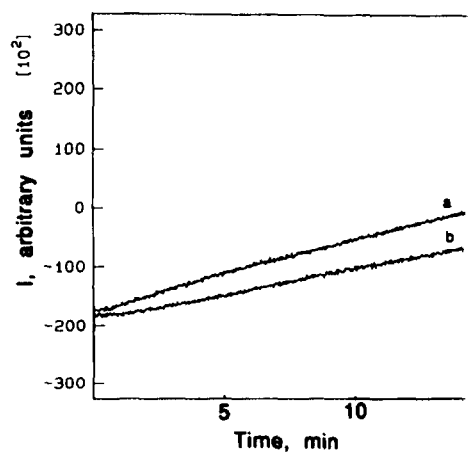

FIG. 6. Comparison of the time dependence of the POBN-HER adduct formation catalyzed by NADPH (a) and NADH (b).

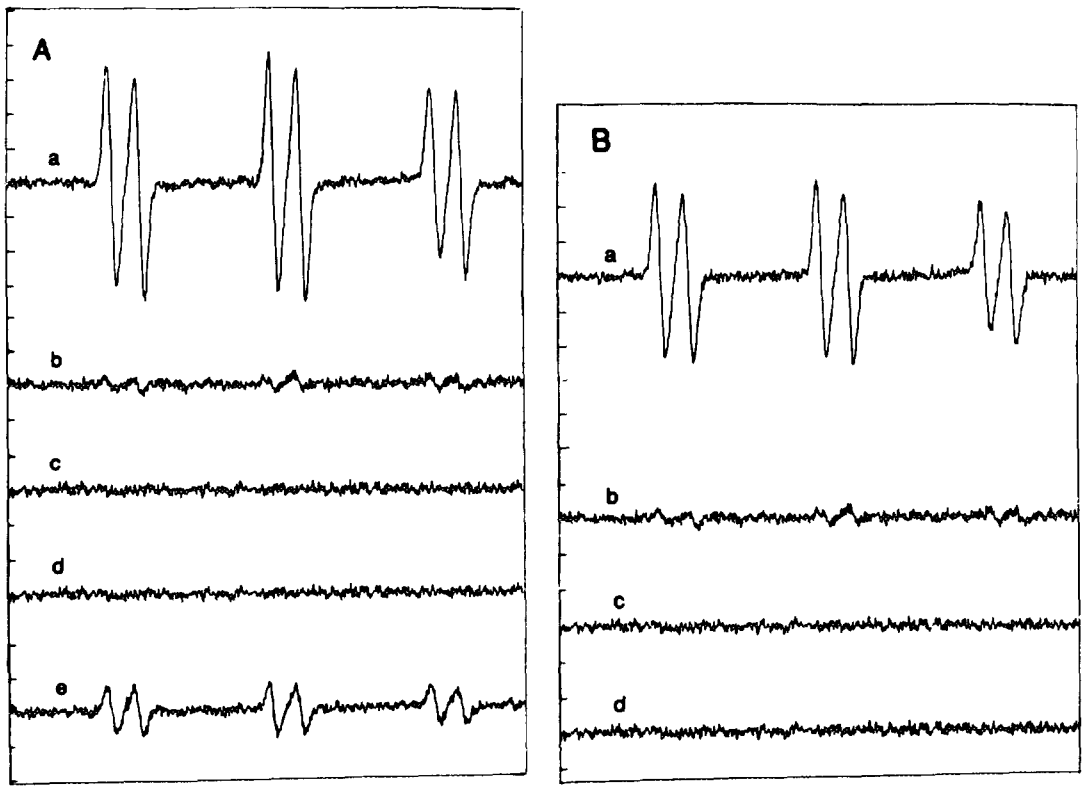

FIG. 7. Controls for the formation of the POBN-HER adduct with NADPH (A) or NADH (B) as cofactor. The reaction system consisted of microsomes (0.3 mg/ml), ferric-EDTA, POBN, ethanol, and either NADPH (A) or NADH (B). (a) No further additions; (b) NADPH (A) or NADH (B) omitted; (c) microsomes omitted; (d) ferric-EDTA omitted but 0.1 mM desferrioxamine added. For the NADPH system, microsomal protein was increased to 1.5 mg per milliliter in the presence of desferrioxamine, and curve (e) was obtained.

complex breakdown while H_2O_2 may arise from the dismutation of superoxide radical or from decay of the peroxygenated cytochrome P450 complex (5, 6, 8, 9). While most studies have used NADPH, the preferred cofactor for the mixed-function oxidase system, as the reductant for microsomal generation of reactive oxygen intermediates, NADH was found to produce H_2O_2 at a rate about 25 to 35% that of NADPH (30). NADH supplies electrons

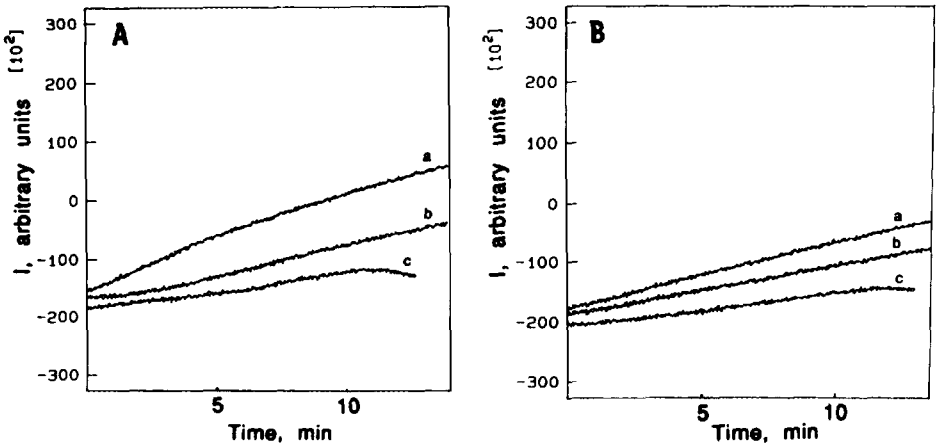

FIG. 8. Effect of different ferric chelates on the formation of the POBN-HER adduct with NADPH (A) or NADH (B) as cofactor. Results show time course kinetics obtained with ferric-EDTA (a), ferric-DTPA (b), and ferric-ATP (c) as the iron catalyst (final concentration of iron of 0.05 mM).

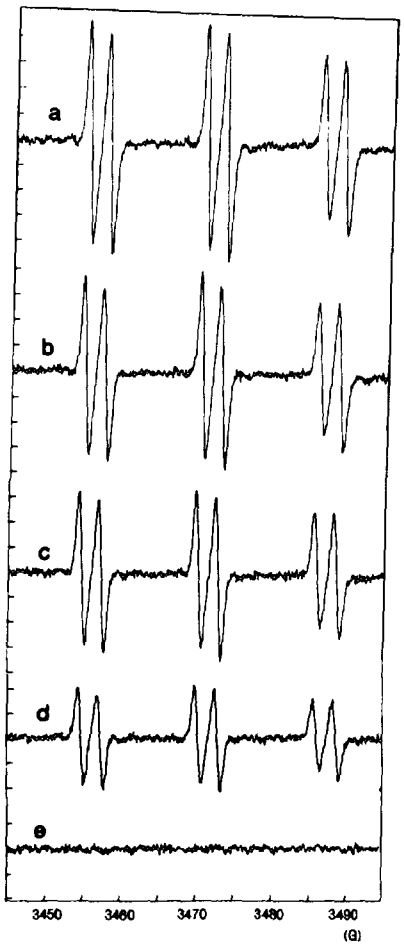

FIG. 9. Effect of anti-oxidants on the formation of the POBN-HER adduct. (a) Incubation (15 min) of microsomes (0.3 mg/ml) with NADPH, ferric-EDTA, 30 mM POBN, and 100 mM ethanol; (b) plus 25 to 100 units per milliliter superoxide dismutase; (c) plus 10 mM GSH; (d) plus 200 mM mannitol; (e) plus 50 units per milliliter catalase.

to NADH-cytochrome b5 reductase, which catalyzes the reduction of cytochrome b5, and these enzymes participate in fatty acid desaturation and elongation (64, 65). These enzymes also may contribute to mixed-function oxidation and NADH synergism of NADPH-supported oxidation by supplying a second electron to the oxygenated cytochrome P450 complex (66, 67). In the current study, NADH was shown to catalyze the production of superoxide by rat liver microsomes and rates with NADH were about 20 to 30% those of NADPH, which is the same magnitude as rates of H_2O_2 production in the presence of the two cofactors. Production of nitroxyl radical from the one electron oxidation of the hydroxylamine by O_2^- was utilized as the method for detection of superoxide (46, 47) since the hydroxylamine is only slowly autooxidizable, has a reasonable rate constant for interacting with superoxide [2.1×10^3 M^{-1} s^{-1}; rate constant for DMPO, 10 M^{-1} s^{-1} (31)], produces a well-defined spectra which can be compared to that of a standard, TEMPO, and does not appear to produce the various artifacts which have been discussed for DMPO, especially in microsomal systems (38–42). Kinetics of the reaction were found to be linear for at least 10 min with both cofactors, and reactions were inhibited more than 80% by superoxide dismutase, validating the role of superoxide in producing the spectral change. Since NADH-cytochrome b5 reductase and cytochrome b5 do not autooxidize to any significant extent, it is likely that cytochrome P450 participates in the NADH-supported production of superoxide and H_2O_2, although this will require validation by experiments with inhibitors and reconstituted systems with the purified enzymes.

In the presence of iron, microsomes can produce 'OH-like species as detected by ESR (32–34) and by oxidation of chemical scavengers (18, 21). Previous results showed that NADH was as effective as NADPH in catalyzing the reduction by microsomes of a variety of ferric complexes (30). In the current study, NADH supported the production of a POBN-HER adduct when microsomes were incubated with POBN and ethanol and a source of iron. The spectrum produced and the splitting constants with microsomes are identical to that produced by NADPH, and are similar to values obtained with chemical systems such as uv irradiation of H_2O_2, the Fenton reaction, or when 'OH is generated by the xanthine oxidase reaction. Production of the POBN–HER adduct requires microsomes, a source of iron, and a reductant, either NADH or NADPH. Several iron complexes can catalyze production of the adduct with ferric EDTA being most reactive, ferric-DTPA being about 60 to 70% as reactive as ferric EDTA and ferric ATP being the least reactive, about 20 to 30% that of ferric EDTA. This pattern of ferric complex effectiveness is the same for NADPH and NADH, and is identical to that previously found for the oxidation of chemical scavengers (16, 17, 30). In this respect, ferric EDTA and DTPA can be directly reduced by NADPH–cytochrome P450 and NADH–cytochrome b5 reductases, while ferric ATP is not an effective substrate for the reductases but requires other microsomal components (e.g., cytochrome P450) for reduction (16, 68, 69). In addition, iron–EDTA is a more effective catalyst of 'OH production than iron–ATP in Fenton-type reactions (14–16, 34). With all three ferric complexes, rates of production of the POBN–HER adduct with NADH as the cofactor are about 60 to 70% those of the NADPH dependent rates.

Production of the POBN–HER adduct is completely prevented by catalase, indicating that H_2O_2 is the precursor of the 'OH. Inhibition is also found in the presence of competitive scavengers such as mannitol and GSH; the latter may react with HER in addition to competing with ethanol for the generated 'OH. The fact that superoxide dismutase is not inhibitory indicates that 'OH is produced largely via a Fenton-type of reaction rather than a Haber–Weiss reaction, most likely reflecting the ability of ferric EDTA to be directly reduced by the reductases, and

therefore obviating a requirement for reduction by superoxide. Superoxide is, however, the likely precursor for H_2O_2 production by the microsomes (5, 6, 9). The lower rates of POBN-HER adduct formation with NADH compared to NADPH are probably due, in part, to the lower rates of superoxide and H_2O_2 production.

Initial experiments indicated that DMPO was not suitable as a spin-trapping agent for experiments designed to compare NADH with NADPH or the effect of different iron complexes in the microsomal system. While the DMPO-OH, DMPO-HER, and DMPO-CH_3 adducts were very stable when $^{\cdot}OH$ was generated by the chemical systems or xanthine oxidase, they were very unstable in the microsomal system. The carbon-centered radical adducts with DMPO were somewhat more stable than the oxygen-centered DMPO-OH adduct as a definite signal could be observed and even followed for about 1 min. However, rapid decay followed making this system unsuitable for accurate kinetics. Rosen and colleagues (38-42) have discussed the practical aspects and problems associated with the use of DMPO, especially in microsomal systems, where bioreduction of the spin-trapped nitroxide rapidly occurs. This rapid decay was not prevented by addition of inhibitors of reductase or P450, or anti-oxidative agents. Although POBN was shown not to decrease total P450 content or convert P450 to P420, some inhibition of microsomal oxidation of substrates such as aminopyrine or ethoxycoumarin by POBN has been observed (70).

Ramos *et al.* (42) and Pou *et al.*[3] recently compared the utility of DMPO, PBN, and POBN for kinetic approaches for spin-trapping of $^{\cdot}OH$, in order to select a spin-trapping system with greater sensitivity and selectivity towards $^{\cdot}OH$ detection. These authors concluded that POBN in conjunction with ethanol was the most sensitive of the spin-trapping systems examined, based upon marked stability, resistance to reduction by superoxide even in the presence of thiols [DMPO-OH and DMPO-CH_3 are degraded by $O_2^{\cdot-}$ (71)], and the greater reactivity of POBN relative to DMPO or PBN with HER (42). POBN appeared to be more satisfactory than DMPO for the detection of $^{\cdot}OH$ in the microsomal system as stable POBN-OH adducts could be observed over a 2- to 3-min incubation period before decay; more significantly, POBN-CH_3 and especially POBN-HER adducts appeared to be quite stable, increasing with time over a 5- to 10-min incubation period and then remaining constant (Fig. 3b). Signal intensity was proportional to microsomal protein, and at lower concentrations of microsomes, e.g., 0.3 mg per milliliter, linear reactions of at least 15 min could be observed. Pou *et al.*[3] observed that in a photolysis system, two POBN adducts with DMSO were produced, one consistent with a carbon-centered radical (POBN-CH_3), the other an oxygen-centered radical (POBN-OCH_3). We also observed similar adducts with POBN plus DMSO when $^{\cdot}OH$ was generated in a Fenton system, but not in a xanthine oxidase system or with microsomes. Production of two types of adducts by photolysis or the Fenton reaction may reflect the production by these systems of large amounts of $^{\cdot}OH$ instantaneously, in contrast to lower steady-state rates of formation of $^{\cdot}OH$ by xanthine oxidase or microsomes. In the case of ethanol, only a carbon-centered radical signal, consistent with the α-hydroxyethyl radical, was detected by all the systems.

In summary, POBN plus ethanol or DMSO was found to be a satisfactory system for kinetic studies on the production of $^{\cdot}OH$ by microsomes, and to allow various comparisons to be made under linear conditions. NADH could support production of superoxide and $^{\cdot}OH$ at rates about 25% and 60 to 70% those of the NADPH-dependent rates. Production of $^{\cdot}OH$ required iron, with ferric EDTA being very reactive while ferric ATP was less reactive. Production of $^{\cdot}OH$ occurred by a Fenton rather than Haber-Weiss type of reaction. The utility of the POBN-HER system for detecting $^{\cdot}OH$ produced by microsomes after chronic ethanol consumption is described in the accompanying manuscript.

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