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Generation of Reactive Oxygen Intermediates by Human Liver Microsomes in the Presence of NADPH or NADH

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SUMMARY

Studies were carried out to evaluate the ability of human liver microsomes to generate superoxide radical and hydrogen peroxide, and to interact with ferric chelates to produce more potent oxidizing species such as the hydroxyl radical (·OH). In the presence of either NADPH or NADH, human liver microsomes produced superoxide and H_2O_2 at rates about 20 to 30% of that found with rat liver microsomes. These lower rates are caused, in part, by the 3-fold lower content of total cytochrome P450 in the human liver microsomes. NADH-dependent rates were about 25 to 30% of the NADPH-dependent rates. In the presence of appropriate ferric complexes, human liver microsomes generated ·OH, promoted cleavage of vicinal diols, and underwent lipid peroxidation. In contrast to results with rat liver microsomes, NADH-dependent rates of ·OH production or lipid peroxidation by human liver microsomes were similar to the NADPH-dependent rates. Human liver microsomes reduced ferric ATP or ferric EDTA at nearly comparable rates with NADPH and NADH. Sensitivity of the various iron-dependent reactions to antioxidants was found to be characteristic of the particular system. These results suggest the possibility that human liver microsomes are an important source of reactive oxygen intermediates, especially under conditions of increased NADH or NADPH availability and elevated iron concentration.

Reactive oxygen species have been implicated in cellular damage and pathogenesis of certain diseases (1–4). In many cases, the initial oxidizing species derived from ground state oxygen is the one-electron reduction product of oxygen, the superoxide anion radical ($O_2^{\cdot-}$), and there has been considerable interest in the superoxide theory of oxygen toxicity (5–8). Superoxide can be produced from radiolysis, from the autooxidation of various chemical and biological agents and metals, from enzymes such as xanthine oxidase, and flavoprotein reductases and organelle electron transport systems (1–7). Isolated liver microsomes produce $O_2^{\cdot-}$ and H_2O_2 during NADPH-dependent electron transfer (9–16). These two reactive oxygen intermediates arise primarily from the decay of oxygenated cytochrome P450 intermediates that are produced in the catalytic mechanism of mixed-function oxidation (13, 14). These initial reactive oxygen species may serve as precursors for the generation of other potent oxidants. In the presence of transition metals such as iron, rat liver microsomes catalyze the production of potent oxidizing agents capable of initiating lipid peroxidation, oxidizing ·OH chemical scavenging agents, and causing cleavage of vicinal diols (17–22). The effectiveness of iron in promoting these reactions is complex and dependent upon the chelated form of the iron as certain iron chelates, which are effective for promoting one reaction pathway, are inhibitory in another pathway. For example, ferric EDTA is a very effective catalyst for microsomal ·OH production, but is inhibitory toward lipid peroxidation or glycerol oxidation to formaldehyde, whereas ferric ATP or ferric histidine show the opposite catalytic effectiveness (18, 21, 22).

Many studies characterizing the generation of reactive oxygen species by cellular organelles have utilized rat liver microsomes. The ability of human liver microsomes to produce $O_2^{\cdot-}$ and H_2O_2 and other potent oxidants, or the interaction of human liver microsomes with different iron complexes and the subsequent generation of potent oxidizing species has not been characterized. It is usually assumed that results with rat liver microsomes on rates of generation of reactive oxygen species, effectiveness of ferric complexes, and sensitivity to antioxidants can be extrapolated to human liver microsomes; however, this remains to be demonstrated. Most studies characterizing the production of reactive oxygen intermediates by microsomes have utilized NADPH as the reductant, because this is the preferred reductant for cytochrome P450-dependent activities (23, 24). NADH-dependent rates of rat liver microsomal lipid peroxidation were only 10 to 30% of the NADPH-dependent

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ABBREVIATIONS: $O_2^{\cdot-}$, superoxide anion radical; ·OH, hydroxyl radical, or a species with the oxidizing potential of the hydroxyl radical; DMSO, dimethylsulfoxide; PNP, paranitrophenol; DMN, *N*-nitrosodimethylamine; SOD, superoxide dismutase; GSH, glutathione; KMB, 2-keto-4-thiomethylbutyric acid; 4-POBN, α[4-pyridyl-1-oxide]-*N*-tert butylnitrone; ESR, electron spin resonance.

rates (25–29). The effectiveness of NADH in interacting with human liver microsomes to catalyze the generation of reactive oxygen intermediates has not been evaluated.

In the current study, experiments were carried out to determine the ability of human liver microsomes to produce $O_2^{\cdot-}$ and H_2O_2, to reduce ferric chelates, to interact with these iron chelates to generate potent oxidizing species, and to evaluate the effect of antioxidants on these reaction pathways. Comparisons between the ability of NADPH and NADH to catalyze production of reactive oxygen species were included to evaluate which is the preferred reductant for the human liver microsomal pathway of oxygen radical generation.

Materials and Methods

Liver microsomes were isolated from male Sprague-Dawley rats weighing about 200 g. The rats were fasted overnight, anesthetized with pentobarbital (150 mg/kg), and sacrificed by decapitation. Three different human livers were obtained from The Mount Sinai transplant program and were kindly provided by Dr. Swan Thung of the Department of Pathology. The livers were from healthy donors who died in accidents; there was no evidence for liver disease in any of the three livers. Liver homogenates (1:10 w/v) were prepared in 0.25 M sucrose, 0.01 M TRIS-HCl, pH 7.4, 0.001 M EDTA, and microsomes were isolated by differential centrifugation. The microsomes were washed twice and resuspended in 0.125 M KCl, 0.01 M potassium phosphate, pH 7.4, and stored in aliquots at −OH70° at a protein concentration of about 10 mg/ml.

The content of cytochrome P450 was determined by the method of Omura and Sato (30), whereas activities of NADPH-cytochrome P450 and NADH-cytochrome b_5 reductase were assayed by following NADPH-dependent reduction of cytochrome *c* or NADH-dependent reduction of ferricyanide, respectively. The oxidation of 0.2 mM PNP to *p*-nitrocatechol or of 4 mM DMN to formaldehyde was determined in a reaction system containing 0.1 M potassium phosphate, pH 7.4, substrate, about 0.1 to 0.3 mg of microsomal protein and 1 mM NADPH to initiate the reaction. After 30 min incubation at 37°, reactions were terminated by the addition of trichloroacetic acid (final concentration of 5% w/v) followed by centrifugation. Concentrations of *p*-nitrocatechol (31) or formaldehyde (32) were determined using extinction coefficients of 9.5 mM^{-1} cm^{-1} and 8.0 mM^{-1} cm^{-1}, respectively, by the indicated colorimetric methods.

Superoxide generation was determined by assaying the production of stable nitroxyl radicals produced from the reaction of hydroxylamines with $O_2^{\cdot-}$ (33, 34). The hydroxylamine, 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine was dissolved in triply distilled, chelex-treated water. Liver microsomes (usually 0.3–0.5 mg/ml) were incubated in 100 mM potassium phosphate buffer, pH 7.4, plus 2.5 mM 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 transferred immediately to a WG-812 Q flat quartz cell and spectra recorded with a Bruker E-300 spectrometer, probe 4102ST, as previously described (35). Kinetics of the reactions 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 obtained with the stable nitroxyl radical, 4-oxo-TEMPO (Aldrich Chem. Co., Milwaukee, WI). Instrument settings for spectra were as follows: sweep width 70 G, sweep time 21 sec, time constant 10 msec, modulation amplitude 0.5 G, modulation frequency 100 kHz, microwave power 11 mW, temperature 20°. The measurement of *g* values was as previously described (34). Under the reaction conditions utilized, e.g., saturating concentration of hydroxylamine, reaction times <10 min, microsomal protein <1 mg/ml, the resulting nitroxyl radical was not significantly reduced back to the hydroxylamine (36). Only the SOD-sensitive formation of nitroxyl radical was used to calculate the rate of $O_2^{\cdot-}$ production.

The production of H_2O_2 was determined by measuring the formation of formaldehyde from the oxidation of methanol by the catalase compound I complex (37). Reactions were carried out in 200 mM KCl, 50 mM TRIS-HCl, pH 7.4, 100 mM methanol, 300 units of catalase, and about 0.5 mg of microsomal protein in a final volume of 100 μl. Reactions were initiated by the addition of NADPH or NADH (1.2 mM final concentration) and terminated by the addition of 30 μl of 20% trichloroacetic acid. The generation of formaldehyde was assayed by the Nash reaction (32).

The ability of human liver microsomes to reduce ferric chelates was determined by assaying for the production of the ferrous 2,2′-dipyridyl complex. The assay mixture contained 0.2 M TRIS-HCl, pH 7.5, 6.5 mM 2,2′-dipyridyl, 1.2 mM NADPH or NADH, 50 μM ferric-100 μM EDTA or 50 μM ferric-1 mM ATP, and about 0.05 mg of microsomal protein in a final volume of 1.0 ml. The rate of reaction was followed at 25° by following the increase in absorbance at 520 nm (38).

Microsomal lipid peroxidation was assayed by measuring the production of TBA reactive metabolites (expressed as malondialdehyde equivalents) (39). Experiments were carried out in 50 mM TRIS-HCl, pH 7.4, 50 μM Fe-ATP, 1.2 mM NADH or NADPH, and 0.3 mg of microsomal protein in a final volume of 100 μl. The reaction was initiated by the reductant and terminated after incubation at 37° for 30 min by addition of 30 μl of 30% trichloroacetic acid. After centrifugation, aliquots of the supernatant were added to an equal volume of TBA (7.3 mg/ml) and heated at 100° for 10 min. After cooling on ice, the absorbance was determined at 535 nm, and the concentration of malondialdehyde was calculated using an extinction coefficient of 156 $mM^{-1}cm^{-1}$ (39).

The oxidation of glycerol to formaldehyde (22) was determined at 37° in a reaction system containing 0.1 M potassium phosphate, pH 7.4, 0.2 M glycerol, 0.01 mM ferric ammonium sulfate, and microsomes equivalent to 1 nmol/ml cytochrome P450 in a final volume of 100 μl. The reaction mixture contained 1 mM sodium azide to inhibit any catalase present as a contaminant. Reactions were initiated by the addition of NADPH to a final concentration of 1.2 mM, and were terminated after 30 min by the addition of trichloroacetic acid (final concentration 6% w/v). Formaldehyde was determined by the Nash procedure (32).

The oxidation of KMB to ethylene gas was used to assay for the production of ·OH or species with the oxidizing power of ·OH (21, 29). KMB oxidation was determined in a reaction system containing 100 mM phosphate buffer, pH 7.5, 30 mM KMB, 1 mM sodium azide, 50 μM iron catalyst, and about 0.7 mg of microsomal protein in a final volume of 1.0 ml. Reactions were initiated by the addition of NADPH or NADH (1.2 mM final concentration) and terminated after 30 min incubation at 37° by addition of 0.33 ml of 1 N HCl. The production of ethylene was determined by head-space gas chromatography.

Detection of ·OH-like species by ESR spectroscopy was carried out by a spin-trapping method, using 4-POBN to form an adduct with the hydroxyethyl radical produced from the interaction of ethanol with ·OH (40–42). Experiments were carried out as previously described (35) in a reaction system containing 0.1 M potassium phosphate buffer, pH 7.4, 0.05 mM ferric EDTA (1:2 chelate), 30 mM 4-POBN, 100 mM ethanol, 0.05 mM sodium azide, and 0.3 to 0.6 mg of microsomal protein in a final volume of 0.5 ml. The reactions were initiated by the addition of either NADPH or NADH to a final concentration 1.2 mM. Kinetics of the reaction were followed by measuring the intensity of the maximum of the 3rd low field line of the 4-POBN-hydroxyethyl adduct. All ESR measurements were carried out at room temperature in a flat quartz cuvette, using a Bruker E-300 spectrometer. The spectra were recorded about 20 sec after initiating the reactions with cofactor. Instrument settings were as described for detection of $O_2^{\cdot-}$.

Where indicated, experiments are from three different preparations of human or rat liver microsomes and results refer to mean ± standard error. Reagents were of the highest grade, and the phosphate buffer and the water were passed through Chelex-100 resin (Bio-Rad, Rich-

mond, CA) to remove metal contaminants. This is especially critical for the $O_2^{\cdot-}$ assay, because in the presence of metals such as iron, hydroxylamines autooxidize. Preliminary experiments validated that the hydroxylamine was not oxidized by any of the reagents. The ferric complexes were prepared by dissolving ferric ammonium sulfate in 0.1 N HCl and then diluting with the appropriate chelating agent. Ferric ATP was used as a 1:20 iron/ATP complex, whereas the other ferric complexes were used as a 1:2 iron/chelator complex.

Results

Mixed-function oxidase activities. The content of cytochrome P450 in human liver microsomes averaged 0.34 nmol/mg of protein in the three samples analyzed, which was about one-third the total P450 content of rat liver microsomes (Table 1). Almost no cytochrome P420 was detected in these samples. The activities of NADPH-cytochrome P450 reductase and NADH-ferricyanide reductase in the human liver microsomes were about one-half the values found for rat liver microsomes (Table 1). As an index of mixed-function oxidase activity, the oxidation of PNP to *p*-nitrocatechol and of DMN to formaldehyde was determined. These compounds are excellent substrates for cytochrome P4502E1 (43, 44). When catalytic activity was expressed on a per mg of microsomal protein basis, oxidation of PNP and DMN were similar between human and rat liver microsomes. When results were expressed on a per nmol of total P450 level, oxidation of the two substrates was 2- to 3-fold higher in the human liver microsomes (Table 1), most likely a reflection of an elevated content of P4502E1 as compared to the noninduced, control rat liver microsomes. These results indicate the presence of active mixed-function oxidase enzymes and mixed-function oxidase catalytic activity in the human liver microsomes.

NADPH- and NADH-dependent production of $O_2^{\cdot-}$ and H_2O_2. Results in Table 2 show that human liver microsomes produce H_2O_2 with either NADPH or NADH as the microsomal reductant. Rates of NADPH- or NADH-dependent H_2O_2 production by human liver microsomes were 4- to 6-fold lower than the rates found with rat liver microsomes when rates were expressed on a per mg of microsomal protein basis; assuming that most of the H_2O_2 produced is P450-dependent, part of the reason for the lower rates of H_2O_2 production by the human liver microsomes may relate to the 3-fold lower content of total cytochrome P450. Rates of NADPH-dependent H_2O_2 generation by human (or rat liver microsomes) were greater than the NADH-dependent rates (Table 2), most likely reflecting the greater effectiveness of NADPH compared to NADH as a reductant for cytochrome P450. There was no effect on the rate of H_2O_2 production by the potent iron chelating agent desferral.

TABLE 1

Mixed-function oxidase activities in human and rat liver microsomes

The various enzyme assays were carried out as described in Methods. Results refer to mean ± standard error and are from three microsomal preparations.

Enzyme assayed	Units	Activity or Content	
		Rat	Human
Cytochrome P450	nmol/mg	1.04 ± 0.19	0.34 ± 0.04
NADPH reductase	μmol/min/mg	0.26 ± 0.04	0.12 ± 0.02
NADH reductase	μmol/min/mg	3.12 ± 0.48	1.89 ± 0.40
PNP hydroxylase	nmol/min/mg	0.70 ± 0.03	0.83 ± 0.27
DMN demethylase	nmol/min/mg	0.42 ± 0.02	0.27 ± 0.04
PNP hydroxylase	nmol/min/nmol of P450	0.68 ± 0.11	2.51 ± 0.53
DMN demethylase	nmol/min/nmol of P450	0.41 ± 0.04	0.81 ± 0.23

TABLE 2

Rates of production of superoxide and H_2O_2 by human and rat liver microsomes

The generation of superoxide or H_2O_2 by human or rat liver microsomes was determined as described in Materials and Methods, using either NADPH or NADH as the microsomal reductant. Results are from three microsomal preparations.

Reaction	Activity	
	Rat	Human
	nmol/min/mg of protein	
NADPH-dependent $O_2^{\cdot-}$	3.0 ± 0.2	0.49 ± 0.04
NADH-dependent $O_2^{\cdot-}$	0.62 ± 0.03	0.17 ± 0.05
NADPH-dependent H_2O_2	4.97 ± 0.36	0.81 ± 0.06
NADH-dependent H_2O_2	0.87 ± 0.08	0.23 ± 0.04

Production of $O_2^{\cdot-}$ was determined from the rate of SOD-sensitive formation of the TEMPO nitroxyl radical, which arises from the interaction of $O_2^{\cdot-}$ with the hydroxylamine substrate (33, 34). Fig. 1, C and D, shows the spectrum of the TEMPO nitroxyl radical produced from the interaction of NADPH or NADH with human liver microsomes. A three-line spectrum with splitting constants identical to the TEMPO standard (A_N, 16.0 G; *g*, 2.0050) was produced; this signal was inhibited more than 80% by SOD (30–50 units/ml, data not shown). Identical ESR signals were produced with rat liver microsomes incubated with either NADPH (Fig. 1A) or NADH (Fig. 1B), although the intensity of the signal was more pronounced with rat liver microsomes. In all cases, little or no signal was produced in the absence of microsomes, reductant or hydroxylamine; 2 to 5 mM hydroxlyamine was generally sufficient for maximal signal intensity (not shown). Desferral (0.02 mM) had no effect on the rate of $O_2^{\cdot-}$ production, indicating the lack of a role for iron. With human liver microsomes, the rate of $O_2^{\cdot-}$ production was linear with at least 2 mg of microsomal protein, whereas linearity was observed with up to about 0.5 mg of rat liver microsomal protein.

By following the increase in intensity of the second line of the triplet spectrum, rates of $O_2^{\cdot-}$ production were linear for at least 10 min of reaction with human or rat liver microsomes and with either NADPH or NADH (data not shown). SOD produced strong inhibition at all time points. Although microsomal reduction of nitroxides such as TEMPO can occur independently of $O_2^{\cdot-}$ (45), the fact that initial rates were determined and that SOD produced greater than 80% inhibition indicates that the reduction of TEMPO was largely due to $O_2^{\cdot-}$. Similar to the rates of H_2O_2 production, rates of $O_2^{\cdot-}$ production by human liver microsomes were 4 to 6 times lower than rates found with rat liver microsomes. NADPH-dependent rates were 3 (human)- or 5 (rat)-fold grater than the NADH-dependent rates (Table 2).

Microsomal lipid peroxidation. The production of TBA-reactive material was measured as an index of lipid peroxidation with ferric ATP as the iron catalyst. In rat liver microsomal systems, initial rates of NADH-dependent lipid peroxidation were 10 to 30% the NADPH-dependent rates (25–29, Fig. 2). However, in human liver microsomes, rates of NADH- and NADPH-dependent lipid peroxidation were similar (Fig. 2). In the absence of ferric ATP, human liver microsomal lipid peroxidation was very low and barely detectable; these low rates are probably caused by small amounts of iron present in the reaction system. The sensitivity of NADPH-dependent human

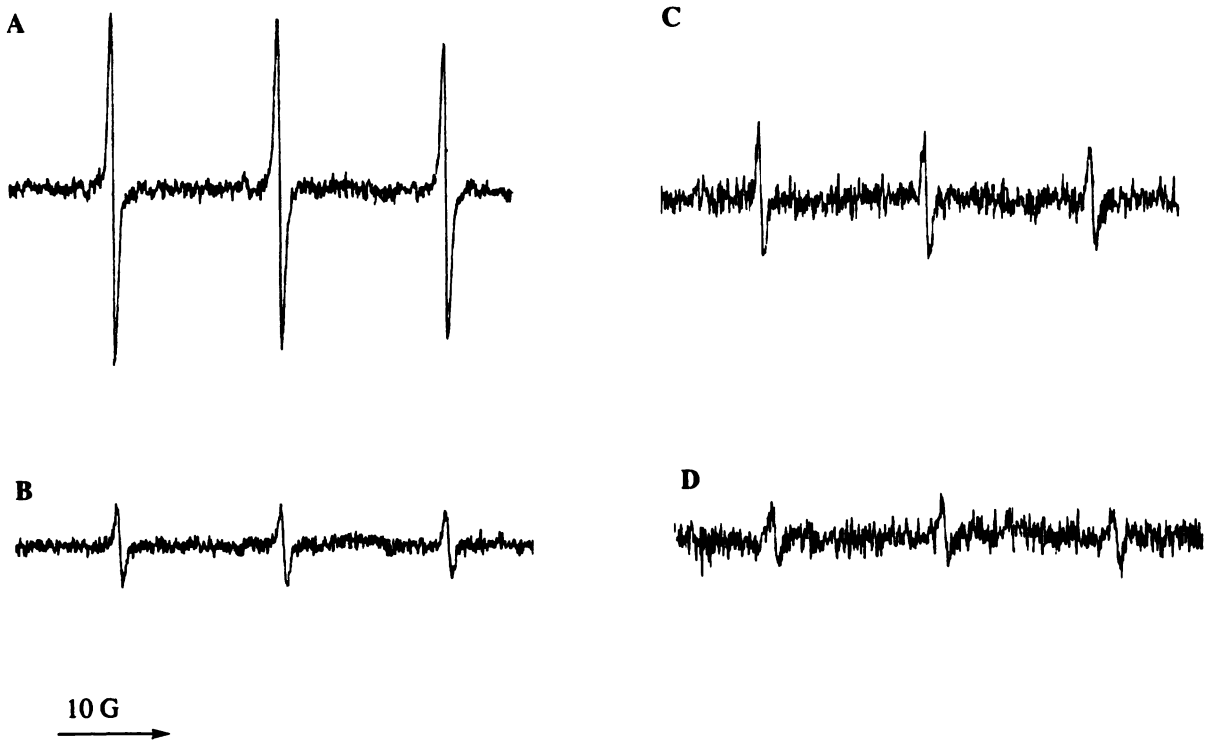

Fig. 1. Production of superoxide by human and rat liver microsomes. The interaction of $O_2^{\cdot-}$ with 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidine was determined as described in Materials and Methods. Experimental conditions were: A, rat liver microsomes, NADPH as reductant; B, rat liver microsomes, NADH as reductant; C, human liver microsomes, NADPH; D, human liver microsomes, NADH. Results are from one out of three different human or rat liver microsomal preparations. The scale (absolute intensity units of the plotted *y* axis) for *A* and *B* is 2000, and that for *C* and *D* is 1000; receiver gain (8×10^3) was constant throughout all experiments.

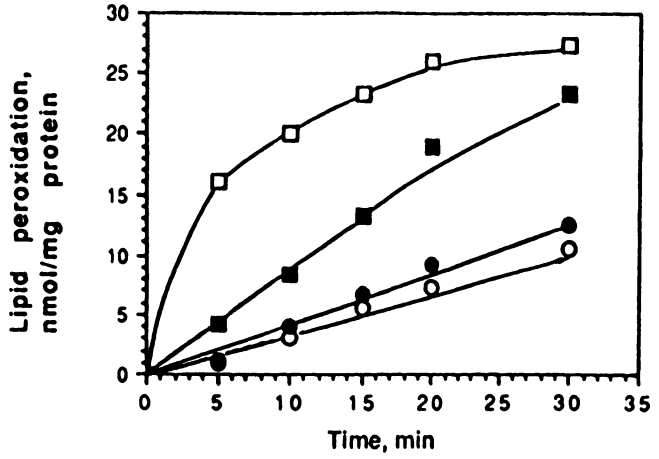

Fig. 2. Production of TBA-reactive material by human and rat liver microsomes. Lipid peroxidation was assayed by the formation of TBA-reactive components as described in Materials and Methods. Reaction conditions were: ○, human liver microsomes, NADPH as reductant; ●, human liver microsomes, NADH as reductant; □, rat microsomes, NADPH; ■, rat microsomes, NADH. Ferric ATP was the iron catalyst to stimulate lipid peroxidation.

TABLE 3

Effect of antioxidants on human liver microsomal production of reactive oxygen species

Lipid peroxidation was carried out using 50 μM ferric ATP as the iron catalyst. The oxidation of glycerol to formaldehyde was determined in the presence of 10 μM ferric ammonium sulfate, whereas the production of ethylene gas from 30 mM KMB was assayed in the presence of 50 μM ferric EDTA as the iron catalyst. Rates of reaction refer to nmol of product (either TBA-reactive material, formaldehyde, or ethylene)/30 min/mg of protein. ND, not determined.

Addition	Rate of reaction and effect of addition			
	Lipid peroxidation NADPH	Glycerol oxidation NADPH	KMB oxidation NADPH	KMB oxidation NADH
	%			
None	7.3	12.1	336	287
SOD, 100 units/ml	7.3 (0)	10.8 (−11)	299 (−11)	227 (−21)
Catalase, 2600 units/ml	6.3 (−14)	4.9 (−59)	77 (−77)	35 (−88)
Ethanol, 50 mM	6.7 (−8)	12.2 (0)	153 (−54)	100 (−65)
DMSO, 50 mM	ND	ND	149 (−55)	120 (−58)
GSH, 5 mM	5.1 (−30)	11.0 (−9)	188 (−44)	187 (−35)
Trolox, 0.1 mM	0 (−100)	12.1 (0)	ND	ND
EDTA, 0.05 mM	1.2 (−84)	1.1 (−91)	ND	ND

liver microsomal lipid peroxidation to antioxidants is shown in the second column of Table 3. SOD, catalase, and ethanol had no effect, which suggests that $O_2^{\cdot-}$, H_2O_2 and, ·OH were not involved in the overall mechanism of lipid peroxidation. GSH was partially inhibitory, whereas trolox completely prevented human liver microsomal lipid peroxidation (Table 3).

Microsomal oxidation of glycerol. Rat liver microsomes and reconstituted systems containing cytochrome P450 oxidized vicinal small molecular weight diols to formaldehyde plus an aldehyde with one less carbon atom (22, 46). This oxidation was mediated by an oxidant derived from the interaction of H_2O_2 with nonheme iron (22, 46). Human liver microsomes also catalyzed an NADPH-dependent oxidation of glycerol to formaldehyde (Table 3, *column 3*). The rate with human liver microsomes is about 6-fold lower than that found with rat liver microsomes (data not shown). Antioxidants such as SOD and ethanol had no effect, whereas catalase was inhibitory toward formaldehyde production, indicating a requirement for H_2O_2, but not $O_2^{\cdot-}$ or ·OH, in the oxidation of glycerol. Antioxidants

that inhibited lipid peroxidation such as trolox had no effect on glycerol oxidation. EDTA strongly inhibited the oxidation of glycerol, which further supports the lack of a role for ·OH in glycerol oxidation because EDTA-chelated iron markedly stimulates microsomal ·OH production (21, 29, and below).

Microsomal ·OH production. In initial experiments, the production of ·OH-like oxidants by human liver microsomes was detected by ESR spectroscopy using 4-POBN as the spin-trapping agent. The primary ·OH radical reacted with ethanol to produce the secondary hydroxyethyl radical, which then reacted with 4-POBN to yield a characteristic six-line signal (40–42). Ferric EDTA was used as the iron catalyst, because this iron complex is very reactive in promoting NADPH- and NADH-dependent production of ·OH-like oxidants with rat liver microsomes (21, 29, 39). Besides ·OH, the interaction of H_2O_2 with ferrous EDTA can give rise to other oxidants, e.g., ferryl species (47, 48), capable of reacting with ethanol to form the hydroxyethyl radical; therefore, production of hydroxyethyl radical may be caused by ·OH and ·OH-like oxidants interacting with ethanol. Fig. 3 shows that incubating human liver microsomes with ferric EDTA, ethanol, 4-POBN, and either NADPH or NADH produced a six-line signal with splitting constants of A_N = 15.67 G and A_H = 2.63 G, similar to values reported in the literature for the 4-POBN-hydroxyethyl adduct (40–42). Signal intensity was similar for NADH and NADPH, and values found with the human liver microsomes were about 1.5 (NADH)- or 2 (NADPH)-fold lower than that found with rat liver microsomes (Fig. 3). Similar experiments were attempted with ferric histidine as the iron catalyst; however, the signal intensity was low, and the high amount of human liver microsomal protein required quenched the reaction and/or caused rapid decay of the 4-POBN-hydroxyethyl adduct signal. Although production of hydroxyethyl radical spin adducts may also be produced by oxygen radical-independent mechanisms (42, 49), no 4-POBN-hydroxyethyl radical spin adduct could be detected in the absence of iron under these conditions.

Subsequent experiments evaluated the production of ethylene gas from KMB as an index for human liver microsomal production of ·OH or of potent oxidants with the oxidizing power of ·OH. A time course for ferric histidine-catalyzed KMB oxidation is shown in Fig. 4. Ethylene production was linear over a 30-min time period and was about 5-fold greater with NADPH as the reductant. With ferric EDTA as the iron catalyst, ethylene production was also linear over a 25-min time period, and similar to the ESR spectroscopy results, rates were similar for NADH and NADPH (Fig. 4). Ferric-EDTA was far superior to ferric histidine as a catalyst for human liver microsomal oxidation of KMB with either NADPH or NADH as reductant.

Results in Table 3 show the effect of anti-oxidants on KMB oxidation by human liver microsomes with either NADPH or NADH as reductant. SOD had little effect on ethylene production; however, catalase and competitive ·OH scavengers lowered the rate of ethylene production. Sensitivity to the various agents was similar with both reductants.

Reduction of iron chelates. A probable initial step in the ability of a ferric chelate to stimulate human liver microsomal production of reactive oxygen intermediates is its reduction to the ferrous redox state. The ability of human liver microsomes to reduce ferric ATP, an effective catalyst for lipid peroxidation, and ferric EDTA, a potent stimulator of ·OH production, was determined. Both ferric chelates were reduced readily by human

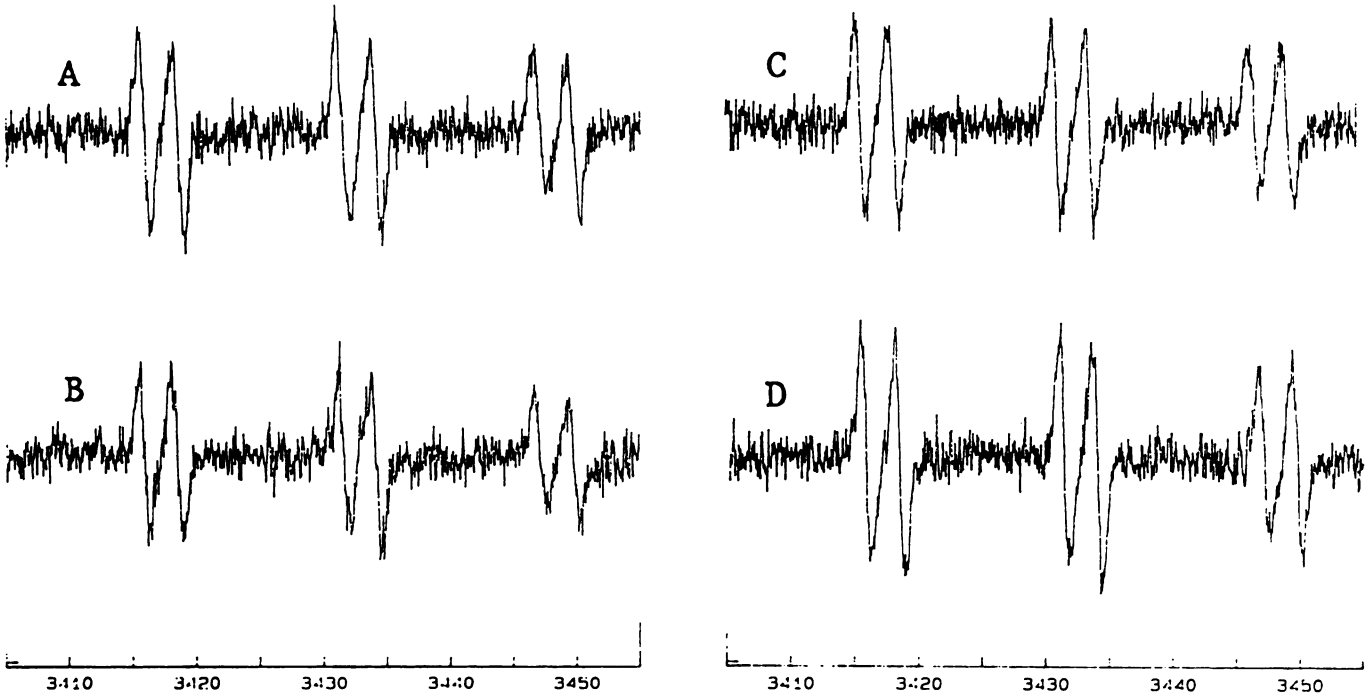

Fig. 3. Production of hydroxyl radical by human and rat liver microsomes. The generation of the hydroxyethyl radical, as a consequence of the interaction of ·OH with ethanol, was determined by spin-trapping with 4-POBN to form the 4-POBN-hydroxyethyl adduct. Ferric EDTA was the iron catalyst to stimulate ·OH production. Experimental conditions were: A, rat liver microsomes, NADPH; B, rat liver microsomes, NADH; C, human liver microsomes, NADPH; D, human liver microsomes, NADH. Protein concentration was 0.30 mg/ml for rat liver microsomes and 0.60 mg/ml for human liver microsomes.

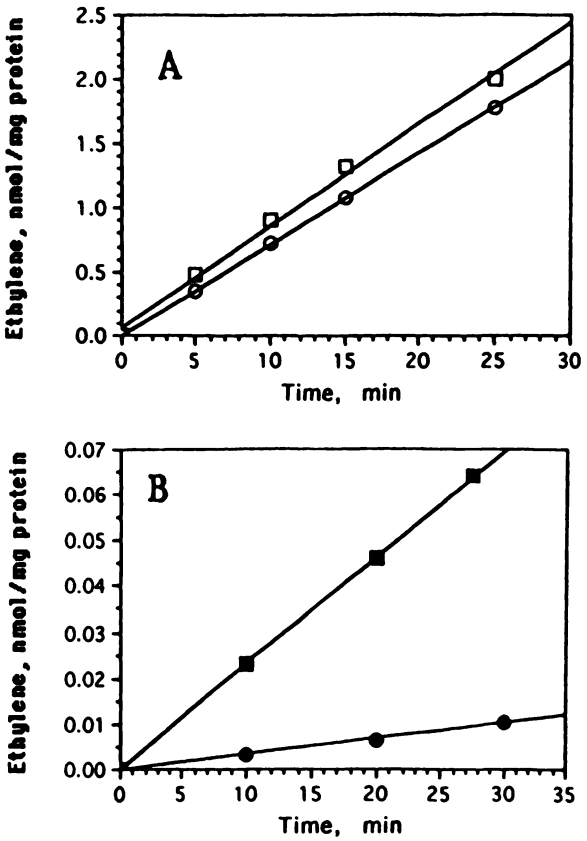

Fig. 4. Oxidation of 2-keto-4-thiomethylbutyric acid to ethylene by human liver microsomes. Experiments were carried out as described in Materials and Methods. A, Ferric EDTA as the iron catalyst and NADPH (□) or NADH (○) as reductants; B, Ferric histidine as the iron catalyst, and NADPH (■) or NADH (●) as the reductant.

TABLE 4

Rates of reduction of iron by human and rat liver microsomes

The reduction of ferric ATP or ferric EDTA in the presence of either NADPH or NADH by human liver and rat liver microsomes was determined as described in Materials and Methods. Results are from three microsomal preparations.

Ferric-chelate	Reductant	Rate of ferrous production	
		Rat	Human
		nmol/min/mg of protein	
Ferric ATP	NADPH	195 ± 21	109 ± 12
Ferric ATP	NADH	161 ± 15	80 ± 9
Ferric EDTA	NADPH	93 ± 10	52 ± 5
Ferric EDTA	NADH	62 ± 5	36 ± 6

liver microsomes with either NADPH or NADH as reductant (Table 4). NADH was about 70% as effective as NADPH, and rates of ferric chelate reduction by the human liver microsomes were about one-half those found with rat liver microsomes (Table 4).

Discussion

Isolated microsomes produce $O_2^{\cdot-}$ and H_2O_2 during the mixed function oxidation cycle (9–16). Superoxide is believed to be produced primarily from the breakdown of the oxycytochrome P450 complex, whereas H_2O_2 is generated from dismutation of $O_2^{\cdot-}$ as well as from the decay of the peroxygenated cytochrome P450 complex (13, 14, 16). Most of these studies demonstrating production of $O_2^{\cdot-}$ and H_2O_2 have utilized rat liver microsomes. In the current study, the ability of human liver microsomes to generate $O_2^{\cdot-}$ and H_2O_2, as well as to interact with iron to generate more potent oxidants, was determined and compared to values obtained with rat liver microsomes. Most previous studies have utilized NADPH as the reductant because this is the preferred cofactor for the microsomal mixed function oxidase system. However, NADH can also support rat liver microsomal production of reactive oxygen intermediates, although at lower rates than NADPH-catalyzed reactions (25–29). Interest in NADH relates to the large increase in the liver NADH/NAD^+ redox ratio as a consequence of the oxidation of ethanol (50, 51). Several of the metabolic effects of ethanol on liver cell function (52–54), including interaction with iron and microsomes (55), have been ascribed to ethanol-derived NADH. Both NADPH and NADH were used as cofactors in experiments evaluating the production of reactive oxygen intermediates from human liver microsomes.

Human liver microsomes generated $O_2^{\cdot-}$ and H_2O_2 with either NADPH or NADH as the reductant. Rates of production of these two reactive oxygen intermediates were about 20% (NADPH) to 30% (NADH) of the values found for rat liver microsomes. Most of the $O_2^{\cdot-}$ and H_2O_2 produced by microsomes is believed to be derived from cytochrome P450, because NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase, and cytochrome b_5 do not auto-oxidize to any significant extent (13, 14, 20, 25, 27, 28, 56–58). It is likely, therefore, that the 3- to 5-fold higher rates of $O_2^{\cdot-}$ and H_2O_2 production by rat liver microsomes compared to human liver microsomes is largely caused by the 3-fold higher content of total cytochrome P450 in rat liver microsomes, although other factors such as the P450 isoform composition and ratios of reductase to cytochromes may also contribute. Levels of P450 isoforms can vary greatly in human liver microsomes (59, 60), and no attempts were made to identify specifically which forms of P450 were present in the samples investigated in this study. NADPH-dependent rates of $O_2^{\cdot-}$ and H_2O_2 production by human liver microsomes were about 3-fold higher than the NADH-dependent rates, which probably reflects the greater effectiveness of NADPH as a cofactor for cytochrome P450-catalyzed reactions. With rat liver microsomes, NADPH-dependent rates of $O_2^{\cdot-}$ and H_2O_2 production were about 5-fold higher than the NADH-dependent rates. Relative effectiveness of NADPH and NADH probably reflects the nature of the P450 composition as well as the molar ratio of reductases to cytochromes in the various microsomal preparations.

To study the interaction of human liver microsomes with ferric chelates, we assayed three reaction pathways that display different characteristics with respect to effectiveness of the ferric-chelate as a catalyst, and to the sensitivity to various anti-oxidants. The reactions were lipid peroxidation, ·OH production, and cleavage of vicinal diols. An initial event in the ability of a ferric chelate to promote these reactions and to redox cycle would be its reduction by the microsomes. Ferric EDTA and ferric ATP were readily reduced by human liver microsomes with either NADPH or NADH as reductant. In fact, rates of reduction of these two ferric chelates (36–109 nmol/min/mg of human liver microsomal protein) were far in excess over the rates of lipid peroxidation, ·OH production, or glycerol oxidation to formaldehyde, suggesting that ferric chelate reduction may not be a rate-determining step in the overall

pathways involved in generation of the various potent oxidizing species.

Human liver microsomes catalyzed the production of TBA-reactive material in the presence of either NADPH or NADH. Ferric ATP was used as the iron catalyst to support lipid peroxidation, because this iron catalyst has been shown to be very effective in many studies demonstrating rat liver microsomal lipid peroxidation (18, 20). Results in Fig. 2 confirm the greater effectiveness of NADPH compared to NADH in catalyzing rat liver microsomal lipid peroxidation. However, with human liver microsomes, rates of NADPH- and NADH-dependent lipid peroxidation were the same, although these rates were lower than corresponding rates with the rat liver microsomes. Cytochrome P450 is usually required for maximal rates of microsomal lipid peroxidation, because the enzyme degrades lipid hydroperoxides and reduces ferric ATP (20, 61). NADH donates electrons to cytochrome P450 *via* NADH-cytochrome b_5 reductase and cytochrome b_5, whereas NADPH donates electrons to P450 directly *via* NADPH-cytochrome P450 reductase or *via* cytochrome b_5. Whether the equal effectiveness of NADH in supporting human liver microsomal lipid peroxidation reflects enhanced interaction with P450 or increased capability of the NADH-cytochrome b_5 system itself to generate the oxidant responsible for lipid peroxidation or to reduce ferric ATP is not clear. NADH was nearly as effective as NADPH in reducing ferric ATP with both human and rat liver microsomes (Table 4); therefore, rates of reduction of iron would not explain the comparable effectiveness of NADPH and NADH in supporting human liver microsomal lipid peroxidation in contrast to the greater effectiveness of NADPH with rat liver microsomes. Recent results suggest that a ferrous-ferric oxygenated complex may be the oxidant responsible for initiation of lipid peroxidation, and a critical balance to maintain the ferrous/ferric redox state is required (19, 62). Perhaps this balance is as appropriately maintained with NADH as with NADPH in human liver microsomes.

The insensitivity of NADPH- or NADH-dependent human liver microsomal lipid peroxidation to catalase, SOD, or ·OH scavenging agents suggests that free $O_2^{\cdot-}$, H_2O_2, and ·OH are not involved in formation of the oxidant responsible for initiating lipid peroxidation. These results are similar to that for rat liver microsomal lipid peroxidation (18, 19, 62, 63).

In the presence of iron, rat liver microsomes can generate an oxidant that cleaves vicinal diols such as glycerol or ethylene glycol (22, 46). Human liver microsomes also can catalyze the oxidation of glycerol to formaldehyde; the lower rate of formaldehyde production by human liver microsomes compared to rat liver microsomes is likely to be caused, in part, by the lower content of P450, because this reaction is dependent upon P450 (22). Some characteristics of the glycerol oxidation reaction are similar to that of lipid peroxidation, e.g., both reactions are inhibited by EDTA, and both reactions are insensitive to SOD or ·OH scavengers such as ethanol. However, the glycerol oxidation pathway, in contrast to lipid peroxidation, is sensitive to catalase but insensitive to trolox. The nature of the oxidant responsible for cleavage of glycerol to formaldehyde is not known, but it is not $O_2^{\cdot-}$, ·OH, or lipid hydroperoxides. The sensitivity to catalase and to EDTA suggests that an oxidant derived from the interaction of H_2O_2 with iron, e.g., a ferryl or preferryl type of oxidant, may be the ultimate oxidizing agent.

Human liver microsomal production of ·OH-like oxidants showed different characteristics than the glycerol oxidation or lipid peroxidation reactions. Ferric EDTA, inhibitory to the latter two reactions, was a powerful catalyst for either KMB oxidation to ethylene or the formation of the 4-POBN-hydroxyethyl adduct. Competitive ·OH scavengers, which had no effect on glycerol oxidation or lipid peroxidation by human liver microsomes, inhibited KMB oxidation. Inhibition by catalase indicates that H_2O_2 is the precursor of the oxidant responsible for KMB oxidation to ethylene. As has been shown for rat liver microsomes (21), ferric EDTA was superior to ferric histidine as a catalyst for human liver microsomal production of ·OH, and with this powerful catalyst, rates of NADPH- and NADH-dependent production of ·OH by human liver microsomes were similar (Fig. 3, Fig. 4A). Ferric EDTA has been shown to be directly reduced by NADPH-cytochrome P450 or NADH-cytochrome b_5 reductase (20, 27); human liver microsomes reduced ferric EDTA with nearly equal effectiveness by NADPH compared to NADH (Table 4). In contrast to ferric EDTA, when ferric histidine was the iron catalyst, NADPH-dependent rates of ·OH production were much greater than the NADH-dependent rates. This could relate to the 3-fold greater rates of H_2O_2 production by human liver microsomes with NADPH as the reductant, and/or to the requirement for cytochrome P450 in ferric-histidine (in contrast to ferric-EDTA) reduction by the microsomes.

In summary, human liver microsomes generate $O_2^{\cdot-}$ and H_2O_2 in the presence of either NADPH or NADH as the microsomal reductant. NADPH-dependent rates are about 3-fold greater than the NADH-dependent reactions. Rates with the human liver microsomes are lower than with rat liver microsomes, which may be caused, in part, by lower cytochrome P450 levels. In the presence of iron chelates, human liver microsomes catalyze the production of ·OH-like species and other potent oxidants capable of initiating lipid peroxidation and oxidizing glycerol. Sensitivity of these various reactions to anti-oxidants and to various ferric complexes is the same for human and rat liver microsomes, although each reaction has its own characteristic profile. These results suggest that human liver microsomes are an important source of reactive oxygen intermediates, especially under conditions of increased cofactor availability and elevated iron concentration.

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