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NADH-DEPENDENT GENERATION OF REACTIVE OXYGEN SPECIES BY MICROSOMES IN THE PRESENCE OF IRON AND REDOX CYCLING AGENTS

ELISA DICKER and ARTHUR I. CEDERBAUM*
Department of Biochemistry, Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

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Abstract—NADH was found previously to catalyze the reduction of various ferric complexes and to promote the generation of reactive oxygen species by rat liver microsomes. Experiments were conducted to evaluate the ability of NADH to interact with ferric complexes and redox cycling agents to catalyze microsomal generation of potent oxidizing species. In the presence of iron, the addition of menadione increased NADPH- and NADH-dependent oxidation of hydroxyl radical (·OH) scavenging agents; effective iron complexes included ferric-EDTA, -diethylenetriamine pentaacetic acid, -ATP, -citrate, and ferric ammonium sulfate. The stimulation produced by menadione was sensitive to catalase and to competitive ·OH scavengers but not to superoxide dismutase. Paraquat, irrespective of the iron catalyst, did not increase significantly the NADH-dependent oxidation of ·OH scavengers under conditions in which the NADPH-dependent reaction was increased. Menadione promoted H_2O_2 production with either NADH or NADPH; paraquat was stimulatory only with NADPH. Stimulation of H_2O_2 generation appears to play a major role in the increased production of ·OH-like species. Menadione inhibited NADH-dependent microsomal lipid peroxidation, whereas paraquat produced a 2-fold increase. Neither the control nor the paraquat-enhanced rates of lipid peroxidation were sensitive to catalase, superoxide dismutase, or dimethyl sulfoxide. Although the NADPH-dependent microsomal system shows greater reactivity and affinity for interacting with redox cycling agents, the capability of NADH to promote menadione-catalyzed generation of ·OH-like species and H_2O_2 or paraquat-mediated lipid peroxidation may also contribute to the overall toxicity of these agents in biological systems. This may be especially significant under conditions in which the production of NADH is increased, e.g. during ethanol oxidation by the liver.

The toxicity associated with redox cycling agents such as menadione or paraquat appears to be related to the generation of reactive oxygen species as a consequence of the one electron reduction of these agents by cellular flavoproteins [1–8]. In microsomes, menadione and paraquat are actively metabolized by NADPH–cytochrome P450 reductase and, in general, NADPH has been shown to be more effective than NADH in mediating redox cycling by menadione and other anthracyclines, and paraquat [1, 3, 4, 9–12]. Iyanagi and Yamazaki [9] showed that menadione is a poor electron acceptor from the purified NADH–cytochrome b_5 reductase, but is more reactive when cytochrome b_5 is added to the reconstituted system. Thor *et al.* [4] demonstrated that the interaction of menadione with NADPH–cytochrome P450 reductase yields a 5-fold greater rate of superoxide production than the interaction of menadione with NADH–cytochrome b_5 reductase. Powis *et al.* [13] showed that in microsomes, the NADPH-dependent rate of superoxide production in the presence of menadione is about twice that of the NADH-dependent rate.

While menadione and paraquat increase microsomal production of superoxide and H_2O_2, the production of more potent oxidizing species which can oxidize various radical scavengers, initiate lipid peroxidation or inactivate enzymes requires catalysis by metals such as iron or copper. The role of iron in promoting microsomal oxygen radical generation is complex as certain ferric complexes [ferric–EDTA, ferric-diethylenetriamine pentaacetic acid (ferric-DTPA)] are effective catalysts for the generation of potent oxidizing species, e.g. hydroxyl radical (·OH)-like species, while other ferric complexes are effective catalysts for microsomal lipid peroxidation and chemiluminescence [14–17]. We had shown previously that the addition of menadione and paraquat increases the oxidation of ·OH scavenging agents by microsomes and NADPH–cytochrome P450 reductase with NADPH as the microsomal reductant [18, 19]. NADH was found to be as effective as NADPH in catalyzing the reduction of various ferric complexes by rat liver microsomes, and in the presence of ferric-EDTA or ferric-DTPA, NADH could support microsomal oxidation of ·OH scavengers and generation of H_2O_2 at rates about one-half those found with NADPH [20]. The studies presented in the current report evaluate the ability of NADH to catalyze synergistic interactions of microsomes with either menadione or paraquat and with various ferric complexes to produce potent oxidizing species capable of oxidizing radical scavengers or initiating lipid peroxidation.

MATERIALS AND METHODS

Liver microsomes were isolated from male,

* Correspondence: Dr. Arthur I. Cederbaum, Department of Biochemistry, Box 1020, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

Sprague–Dawley rats which consumed 0.1% (w/v) sodium phenobarbital in their drinking water for 2 weeks. The microsomes were isolated by differential centrifugation, washed twice with 0.15 M KCl at 100,000 *g*, and stored at −70°.

Microsomal generation of ·OH-like species was determined by assaying for the production of ethylene gas from 10 mM 2-keto-4-thiomethyl butyric acid (KMB) or of acetaldehyde from 50 mM ethanol [21]. The term ·OH or ·OH-like species is utilized for simplicity although it is recognized that these scavengers are not specific for interaction only with ·OH. The basic reaction system consisted of 100 mM potassium phosphate buffer, pH 7.4, about 0.2 mg microsomal protein, 1 mM sodium azide to inhibit catalase, and 10 mM KMB or 50 mM ethanol in a final volume of 1 mL. In most experiments 50 μM ferric-EDTA was added as the iron catalyst. Reactions were initiated by the addition of NADH to a final concentration of 2 mM and terminated at various time points by the addition of 1 mL of 1 N HCl. In some experiments, 2 mM NADPH replaced NADH as the microsomal cofactor. Ethylene or acetaldehyde was determined by a head space gas chromatography procedure [21]. All values were corrected for zero-time controls in which HCl was added prior to the NADH or NADPH.

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 [22]. Reactions were carried out as previously described [20]. The production of thiobarbituric acid (TBA) reactive material was assayed as an index of microsomal lipid peroxidation. Experiments were performed as previously described [20], and the production of malondialdehyde equivalents was determined from the absorbance at 535 nm, using a millimolar extinction coefficient of 156 [23].

The ferric complexes were prepared by dissolving ferric-ammonium sulfate in 0.1 N HCl, and then diluting with the respective chelator to the appropriate stock concentration. Ferric-ATP was utilized as a 1:20 complex, whereas the other ferric complexes were all utilized as a 1:2 complex. All results are from experiments performed in duplicate, and replicated with at least two different microsomal preparations. Differences between preparations generally did not exceed 10%.

Menadione, paraquat, NADH, NADPH, KMB, and most other biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO. Ferric ammonium sulfate and buffers were from Fisher Scientific, Springfield, NJ.

RESULTS

NADH-dependent oxidation of ·OH scavenging agents. Initial experiments employed ferric-EDTA as the iron catalyst since this complex was the most effective in catalyzing NADPH- and NADH-dependent production of ·OH-like species by microsomes [17, 20]. In the absence of ferric-EDTA, rates of ethylene production were very low with either NADH (Fig. 1A) or NADPH (Fig. 1B) as cofactors, and the addition of either menadione or paraquat had little or no effect (Fig. 1). In the presence of ferric-EDTA, microsomes catalyzed a time-dependent oxidation of KMB with either NADH (Fig. 1A) or NADPH (Fig. 1B) as cofactors; the NADPH-dependent rate of ethylene production was about 3-fold greater than the NADH-dependent rate. With NADH as cofactor, the addition of menadione resulted in a 3-fold increase in ethylene production, whereas paraquat had no effect (Fig. 1A). With NADPH as cofactor, both menadione and paraquat produced striking increases in the oxidation of KMB at shorter reaction times (Fig. 1B). Under all reaction conditions, little or no ethylene was produced in the absence of microsomes, KMB or cofactor, or in zero-time controls in which acid was added prior to initiation of the reaction with reductant. Essentially similar results were obtained with other ·OH scavengers, e.g. oxidation of ethanol to acetaldehyde. With either ethanol or KMB as substrate, maximal stimulation occurred at menadione concentrations of 0.05–0.10 mM,

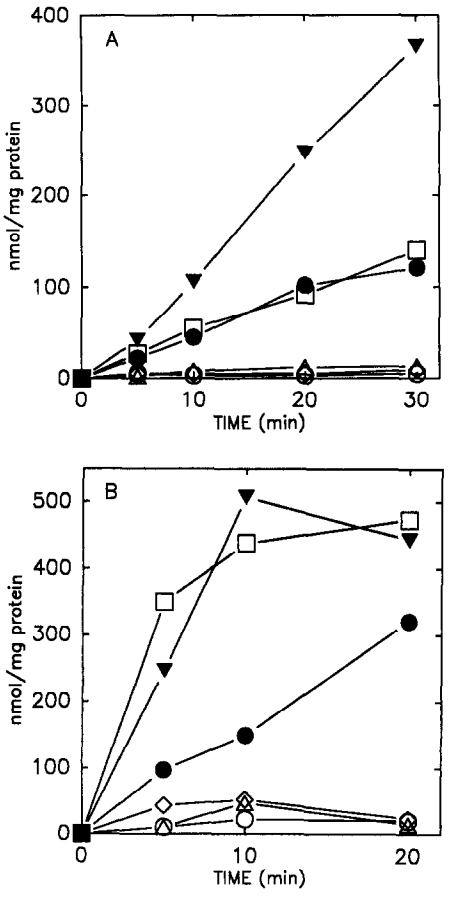

Fig. 1. Effect of menadione or paraquat on NADH-dependent (A) or NADPH-dependent (B) oxidation of KMB to ethylene. Experiments were performed as described under Materials and Methods, under the following conditions: (○) control; (△) 0.1 mM menadione; (◇) 2 mM paraquat; (●) 50 μM ferric-EDTA; (▼) ferric-EDTA plus menadione and (□) ferric-EDTA plus paraquat. Results are from two microsomal preparations.

Table 1. Effects of radical scavengers on NADH-dependent oxidation of KMB

Addition	Rate of ethylene production (nmol/min/mg microsomal protein)		
	Ferric-EDTA	Ferric-EDTA + Menadione	Ferric-EDTA + Paraquat
None	7.86	32.00	10.11
SOD	6.76 (−14)	29.77 (−9)	9.51 (−6)
Catalase	1.27 (−84)	0.88 (−97)	1.28 (−87)
DMSO	2.54 (−67)	10.10 (−68)	3.56 (−65)
Ethanol	3.48 (−56)	13.42 (−58)	4.99 (−51)

The NADH-dependent oxidation of KMB to ethylene by microsomes was determined as described under Materials and Methods in the presence of 50 μM ferric-EDTA alone or ferric-EDTA plus 0.1 mM menadione or ferric-EDTA plus 2 mM paraquat. SOD and catalase were present at 43 and 1300 units per mL, respectively, while final concentrations of DMSO or ethanol were each 50 mM. The numbers in parentheses refer to the percent inhibition by the addition. Results are from experiments performed with two microsomal preparations.

whereas paraquat, even at 2 mM, produced only a 40% increase in NADH-dependent ·OH production (data not shown).

Effects of radical scavengers. The NADH-dependent oxidation of KMB in the absence or presence of either menadione or paraquat displayed identical sensitivity to various antioxidative agents. Superoxide dismutase (SOD) had no effect, whereas catalase produced potent inhibition (Table 1). Competitive ·OH scavenging agents such as dimethyl sulfoxide (DMSO) or ethanol also caused inhibition of ethylene production from KMB. The strong inhibition by catalase suggests that H_2O_2 is required as a precursor for the oxidant responsible for the oxidation of KMB, and that H_2O_2 plays a role in the augmented rates of KMB oxidation found in the presence of menadione.

Effects of various ferric complexes. With either NADH or NADPH as cofactor, ferric complexes such as ferric-EDTA or ferric-DTPA were found to be very effective in catalyzing the generation of ·OH-like species by microsomes, whereas other iron complexes such as ferric ammonium sulfate, ferric-ATP, or ferric-citrate were much less reactive [17, 24]. Results in Table 2 show that the highest rates of NADH-dependent oxidation of KMB or ethanol occurred in the presence of ferric-EDTA or ferric-DTPA. Paraquat, which was ineffective in stimulating the oxidation of KMB or ethanol with ferric-EDTA as catalyst, was also ineffective with ferric-DTPA, ferric ammonium sulfate, ferric-ATP, and ferric-citrate as iron catalysts. Menadione, which was effective with ferric-EDTA as the iron catalyst, also was very effective in stimulating NADH-dependent microsomal generation of ·OH-like species with all the ferric complexes evaluated (Table 2). Indeed, the percent increase in microsomal oxygen radical generation produced by menadione with ferric-ATP or ferric-citrate exceeded or was at least as great as the percent increase produced by menadione with ferric-EDTA or ferric-DTPA as iron catalysts.

Microsomal production of H_2O_2. The potent inhibition of the NADH-dependent oxidation of ·OH scavengers by catalase in the absence or presence of menadione or paraquat suggests a critical role for H_2O_2 in the overall reaction mechanism. In the absence of added iron, NADH-dependent generation of H_2O_2 was relatively low and the addition of paraquat produced less than a 2-fold increase (Table 3). Menadione, at concentrations that stimulated NADH-dependent microsomal production of ·OH-like species, strikingly increased NADH-dependent microsomal H_2O_2 generation (Table 3). In the presence of ferric-EDTA, rates of H_2O_2 production were enhanced markedly compared to the rates in the absence of added iron. Although it would be expected that ferric-EDTA should decompose the H_2O_2 to ·OH, large amounts of catalase plus methanol were present in the H_2O_2 detection system to compete with the ferric-EDTA for the generated H_2O_2. Menadione, but not paraquat, produced a further increase in these already elevated rates of H_2O_2 production (Table 3). With ferric-ATP as the added iron catalyst, rates of H_2O_2 production were relatively low and were similar to the rates found in the absence of added iron. Paraquat produced only a slight increase, whereas menadione strikingly elevated the rate of H_2O_2 production (Table 3). With NADPH as the microsomal reductant, paraquat was as effective as menadione in increasing H_2O_2 generation in the absence or presence of added iron (Table 3).

NADH-dependent microsomal lipid peroxidation. The production of thiobarbituric acid reactive material was taken as a reflection of microsomal lipid peroxidation. Ferric-ATP was utilized initially as the iron catalyst in view of its known effectiveness with NADPH [14, 15] and NADH [20] as cofactor. NADH-dependent microsomal lipid peroxidation in the presence of ferric-ATP was linear for about 10 min (Fig. 2). Menadione completely inhibited microsomal lipid peroxidation (Fig. 2), in contrast to the stimulatory effect it had on microsomal ·OH and H_2O_2 production. Paraquat produced about a 2-fold increase in NADH-dependent microsomal lipid peroxidation (Fig. 2).

The catalytic effectiveness of various ferric complexes for NADH-dependent microsomal lipid peroxidation is opposite to that for NADH-

Table 2. Effects of ferric chelates on NADH-dependent oxidation of KMB or ethanol by microsomes

Substrates	Ferric chelate	Rate of oxidation (nmol/min/mg protein)			Stimulation (%) by	
		No addition	Menadione	Paraquat	Menadione	Paraquat
KMB	Ferric-NH_4SO_4	1.19	5.06	1.46	325	23
	Ferric-ATP	0.72	6.46	1.20	797	67
	Ferric-citrate	0.93	6.57	1.30	606	40
	Ferric-DTPA	5.02	24.94	9.49	397	89
	Ferric-EDTA	8.00	33.60	10.02	320	25
Ethanol	Ferric-NH_4SO_4	2.08	4.46	3.12	114	50
	Ferric-ATP	2.51	7.18	3.23	184	29
	Ferric-citrate	2.96	11.31	4.36	282	47
	Ferric-DTPA	12.24	42.12	21.55	244	76
	Ferric-EDTA	15.53	59.46	18.03	283	16

The NADH-dependent oxidation of 10 mM KMB to ethylene or of 50 mM ethanol to acetaldehyde by microsomes was determined as described under Materials and Methods in the presence of the indicated ferric chelate, added to a final concentration of 50 μM. When present, the final concentrations of menadione or paraquat were 0.1 or 2 mM, respectively. Results are from experiments performed with two microsomal preparations.

Table 3. Comparison of the effects of menadione and paraquat on NADH and NADPH microsomal production of H_2O_2

Ferric complex	Addition	Rate of H_2O_2 production (nmol/min/mg protein) NADH	NADPH
None added		0.89 ± 0.31	4.40
	Menadione	13.42 ± 3.17	36.83
	Paraquat	1.74 ± 0.35	34.45
Ferric-EDTA		14.38 ± 2.50	14.73
	Menadione	27.88 ± 5.40	34.47
	Paraquat	14.11 ± 3.94	29.27
Ferric-ATP		0.89 ± 0.36	3.35
	Menadione	7.98 ± 2.54	22.90
	Paraquat	1.10 ± 0.29	19.98

The generation of H_2O_2 by microsomes was determined as described under Materials and Methods in the presence of either NADH or NADPH as cofactor. When present, final concentrations of menadione or paraquat were 0.1 and 2 mM, respectively. Results are from five experiments for NADH as cofactor (expressed as the mean + SEM) and from two experiments for NADPH as cofactor.

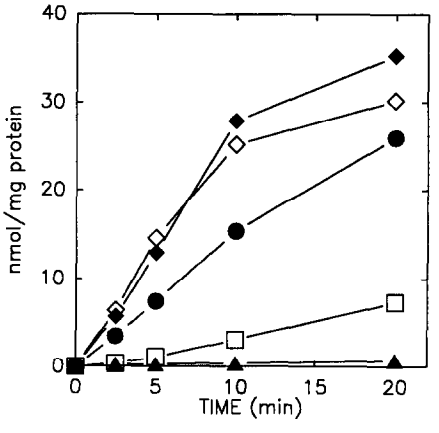

Fig. 2. Effect of menadione or paraquat on NADH-dependent microsomal lipid peroxidation. Experiments were performed as described under Materials and Methods. Values refer to nmol TBA-reactive material produced per mg protein. Ferric-ATP (50 μM) was added as the iron catalyst. Key: (●) control; (□) 0.02 mM menadione; (▲) 0.1 mM menadione; (◆) 1 mM paraquat; and (◇) 2 mM paraquat. Results are from two microsomal preparations.

dependent microsomal generation of ·OH-like species [20]; Table 4 confirms the effectiveness of ferric-ATP and ferric-citrate, and the inability of ferric-EDTA, and ferric-DTPA, to catalyze NADH-dependent microsomal lipid peroxidation. Menadione was a strong inhibitor of lipid peroxidation irrespective of the iron catalyst, whereas paraquat produced a 2-fold increase when ferric ammonium sulfate or ferric-citrate or ferric-ATP was the iron catalyst; no increases were observed in the presence of paraquat plus either ferric-EDTA or ferric-DTPA (Table 4).

The lack of a role for ·OH-like species in initiating NADPH-dependent microsomal lipid peroxidation has been emphasized [14, 15, 17, 25, 26]. The opposing catalytic effectiveness of ferric-ATP and ferric-EDTA on lipid peroxidation and ·OH production suggests that free ·OH is not involved in NADH-dependent microsomal lipid peroxidation. In support of this, the ferric-ATP catalyzed, NADH-dependent microsomal production of TBA-reactive material was insensitive to superoxide dismutase, catalase, and DMSO (Table 4). Moreover, the increase in lipid peroxidation produced by the addition of paraquat was also insensitive to these anti-oxidative agents (Table 4).

Table 4. Effects of menadione and paraquat on NADH-dependent microsomal lipid peroxidation

Ferric chelate	Addition	Rate of production of TBA-reactive material (nmol/min/mg microsomal protein)		
		Control	Menadione	Paraquat
None added		0	0.04	0
Ferric-NH_4SO_4		0.73	0.21	1.63
Ferric-EDTA		0	0.02	0
Ferric-DTPA		0	0.02	0.02
Ferric-citrate		1.14	0.06	2.31
Ferric-ATP		1.41	0.40	2.64
Ferric-ATP		1.23	0.08	2.59
Ferric-ATP	SOD	1.28	0.18	2.36
Ferric-ATP	Catalase	1.43	0.14	2.77
Ferric-ATP	DMSO	1.23	0.11	2.59

The NADH-dependent production of TBA-reactive material by microsomes was determined as described under Materials and Methods in the presence of the indicated ferric chelate, added to a final concentration of 50 μM. When present, final concentrations of menadione or paraquat were 0.1 and 2 mM, respectively. SOD and catalase were present at 43 and 1300 units per mL, respectively, while the final concentration of DMSO was 50 mM. Results are from experiments performed with two microsomal preparations.

DISCUSSION

In the presence of NADPH, redox cycling agents such as menadione or paraquat increase the production of a variety of reactive oxygen intermediates by microsomes [1–13]. With NADH as the electron donor, menadione was found to be effective in catalyzing the production of ·OH-like species by microsomes and to elevate the production of H_2O_2. Enhancement of pyridine nucleotide oxidation, oxygen uptake, and superoxide production by quinones is generally more effective with NADPH than NADH as the reducing agent for the microsomal electron transfer chain [1, 3, 4, 9–12]. At high concentrations (0.05 to 0.10 mM), the increase in production of ·OH-like species or H_2O_2 generation by menadione in the presence of NADH is nearly equivalent to the increases found in the presence of NADPH. In contrast to results with menadione, paraquat, even at very high concentrations, was ineffective in stimulating NADH-dependent microsomal production of ·OH-like species or of H_2O_2. Paraquat has been shown to be relatively unreactive with NADH, compared to NADPH, in a variety of biological systems [11, 12, 27]. The relative ineffectiveness of paraquat in microsomal systems with NADH most likely is a consequence of the higher redox potential of NADH–cytochrome b_5 reductase as compared to NADPH–cytochrome P450 reductase [9, 28].

Superoxide dismutase has no effect on the NADH-dependent production of ·OH-like species or in the increase produced by menadione, suggesting that NADH–cytochrome b_5 reductase rather than superoxide reduces the ferric chelates. The potent inhibition by catalase of ethylene production under all conditions indicates that H_2O_2 serves as the precursor of the oxidant responsible for oxidizing KMB, and eliminates a mechanism for the enhancement of ethylene generation by menadione based upon direct oxidation of KMB by the menadione semiquinone (or paraquat) radical. A likely explanation for the increase in NADH-dependent microsomal production of ·OH-like species by menadione may reflect increases in the rate of H_2O_2 generation by menadione. Rates of H_2O_2 production may be rate-limiting for the overall oxidation of KMB in the presence of reactive iron catalysts such as ferric-EDTA; indeed rates of generation of ·OH-like species were similar to the rates of H_2O_2 generation (Tables 1–3). Conversely, paraquat, which failed to stimulate NADH-dependent ·OH production, also failed to increase NADH-dependent H_2O_2 generation; paraquat was effective in stimulating both NADPH-dependent production of ·OH-like species and H_2O_2 generation.

In the absence of added iron, menadione and paraquat had little effect on NADH or NADPH microsomal production of ·OH-like species, indicating that there was little or no direct interaction of the menadione semiquinone or paraquat radical with H_2O_2 to yield ·OH. Rates of NADH-dependent production of ·OH-like species in the presence of menadione or paraquat were highest in the presence of ferric-EDTA or ferric-DTPA (Table 2). However, other iron complexes such as ferric ammonium sulfate, ferric-ATP, or ferric-citrate, that are not very active in stimulating NADH-dependent microsomal ·OH production, become considerably more effective in the presence of menadione. The increased effectiveness of all these ferric complexes in catalyzing production of ·OH-like species in the presence of NADH and menadione may reflect increasing rates of reduction of the ferric complex by the one electron reduced radical [29, 30], and the

increase in H_2O_2 generation. The ability of menadione to interact with physiologically relevant ferric complexes such as ferric-ATP or ferric-citrate and synergistically enhance the NADH- (and NADPH-) dependent generation of ·OH-like species by the microsomes may be important in contributing towards the overall toxicity and development of oxidative stress produced by menadione and quinones in biological systems. Paraquat, which is not effective in promoting NADH-dependent ·OH generation with ferric-EDTA, remained unreactive with all the other ferric complexes evaluated.

Menadione has been shown to inhibit NADPH-dependent microsomal lipid peroxidation [30, 31]; NADH-dependent production of thiobarbituric acid reactive material was also very sensitive to inhibition by menandione irrespective of the iron complex (Table 4). It has been suggested that the menadione semiquinone radical may quench propagation steps associated with the lipid peroxidation process [30, 31]. Paraquat has been reported to increase, decrease, or to have no effect on NADPH-dependent microsomal lipid peroxidation [6, 8, 32–36]. We observed that paraquat produced a modest 2-fold raise in NADH-dependent lipid peroxidation. Because paraquat is not very effective in stimulating NADH-dependent production of ·OH-like species, the increase in lipid peroxidation is not likely due to an ·OH-dependent reaction. Lipid peroxidation, in the absence or presence of paraquat, is not sensitive to catalase or DMSO. The oxidants responsible for microsomal lipid peroxidation and oxidation of ·OH scavengers are clearly not the same [14, 15, 17, 25, 26]. Recent results suggest that lipid peroxidation may be initiated by a ferrous-ferric oxygenated complex, and maximal rates are obtained when there is an equal balance between the ferrous and ferric redox state [37–40]. Since iron is required for the stimulation by paraquat (Table 4), direct decomposition of preformed lipid hydroperoxides by paraquat radical is not a likely reason for the stimulation of lipid peroxidation by paraquat. No stimulation was found under anaerobic conditions, suggesting that paraquat was stimulating initiation of lipid peroxidation rather than breakdown of preformed lipid hydroperoxides.

The greater reactivity and apparent affinity of menadione, and especially paraquat, with NADPH as the microsomal reductant, suggests that this cofactor is primarily responsible for the generation of reactive oxygen species and the toxicity mediated by these redox recycling agents. The capability of NADH to promote menadione-catalyzed generation of ·OH-like species or paraquat-mediated lipid peroxidation may also contribute to the overall toxicity of these agents, especially under conditions in which the supply of NADPH may become limiting. Our interest in the ability of NADH to promote microsomal oxidation of ·OH scavengers, H_2O_2 generation, and lipid peroxidation involves the possible role such interactions may contribute towards the hepatotoxic actions of ethanol. Normally, the cytosolic redox state is highly oxidized and oxidation of ethanol by alcohol dehydrogenase results in a marked decrease in the hepatic NAD/NADH redox ratio [41, 42]. Several of the toxic actions of ethanol to the liver have been ascribed to this redox state change [43–45]. Increased and prolonged availability of NADH derived from the oxidation of ethanol may contribute to the development of oxidative stress to the liver and thereby play a role in ethanol hepatotoxicity. From a practical point of view, the synergistic interactions of NADH, microsomes, and menadione may be important if such interactions also occur with other quinone redox cycling agents of clinical significance, e.g. Adriamycin® and mitomycin C.

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