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Inhibition of Microsomal Lipid Peroxidation by Naphthoquinones: Structure–Activity Relationships and Possible Mechanisms of Action[1]

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Menadione (2-methyl-1,4-naphthoquinone) is a remarkably potent inhibitor of microsomal lipid peroxidation, effective at submicromolar concentrations. Its possible mechanism of action and the relationship between naphthoquinone structure and antioxidant activity were the topics of this investigation. In the microsomal lipid-peroxidizing system dependent on NADPH and ferric pyrophosphate, menadione, at concentrations of 50 μM or higher virtually eliminated the accumulation of malondialdehyde and lipid hydroperoxides. In the NADPH-independent, cumene hydroperoxide-dependent system, menadione was also an effective antioxidant, but only in the presence of reducing equivalents. These and other observations indicate that a reduced form of menadione, either the hydroquinone or semiquinone, is the active antioxidant, and suggest that it may trap hydroperoxy radicals, alkoxy radicals, or other free radicals involved in propagating lipid peroxidation. Moreover, these results show that electron diversion per se cannot account for the antioxidant effects of menadione. A comparison of the antioxidant activities of eight 1,4-naphthoquinones indicated that methyl substitution of C-2, lack of steric hindrance at C-3 or C-5, and (in the case of weak acids) a relatively high pK_a are favorable structural features associated with strong antioxidant activity.

Microsomal lipid peroxidation, a process that may contribute to the toxicity of many xenobiotics (1), has been studied extensively since its discovery two decades ago (2). Much attention has been focused on the role of the microsomal electron transport system (3-5), chelated iron (6, 7), reduced oxygen (8-10), and organic hydroperoxides (7, 11) in the initiation and propagation of the peroxidative reaction. Present understanding of the events in the reaction sequence derives principally from studies performed in two model systems. The first of these requires NADPH, NADPH cytochrome *P*-450 reductase, and chelated iron, and is activated by the reduction of the iron to an oxygen-binding species (6, 7). The ferrous-oxygen complex rearranges to perferryl ion, which is believed to initiate lipid peroxidation by abstracting a methylene hydrogen from an unsaturated fatty acid, creating an alkyl radical that combines with oxygen to form a lipid hydroperoxide (6). The lipid hydroperoxide then serves as a source of other free radicals as the chain reaction is propagated by iron species such as ferric cytochrome *P*-450 (7). In the second model system, the requirements for reducing equivalents and non-heme iron are bypassed by initiating the reaction with

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an organic hydroperoxide such as cumene hydroperoxide (7, 11). In this system, initiation is accomplished when the unsaturated fatty acid donates a hydrogen to the microsomal peroxidase, ferric cytochrome *P*-450 (11, 12).

In the presence of sufficient butylated hydroxytoluene (13, 14), glutathione (14, 15), and other antioxidants (1, 15), microsomal lipid peroxidation does not occur and the function of the microsomal electron transport system is preserved. Menadione (2-methyl-1,4-naphthoquinone) is an especially potent antioxidant, as it confers complete protection at submicromolar concentrations (16), but the mechanism of its action is not well understood; this was addressed in a series of experiments described in this paper.

MATERIALS AND METHODS

Chemicals. NADPH, TRIZMA base, hydrogen peroxide, and thiobarbituric acid were purchased from Sigma Chemical Company (St. Louis, Mo.); cumene hydroperoxide, menadione, and silver oxide were purchased from Pfaltz and Bauer, Inc. (Stamford, Conn.); methyl iodide, sodium peroxide, palmitoyl chloride, and 5-hydroxy-2-methyl-1,4-naphthoquinone were from Aldrich Chemical Company (Milwaukee, Wisc.); chromium trioxide and potassium iodide were from Mallinckrodt, Inc. (Paris, Ky.); 2,3-dimethylnaphthalene and 1,4-naphthoquinone were from Fluka Chemical Corporation (Hauppauge, N. Y.); and ferric pyrophosphate was obtained from K and K Laboratories, Inc. (Plainview, N. Y.) All organic solvents were reagent grade, from Mallinckrodt.

Eight derivatives of 1,4-naphthoquinone were tested as inhibitors of microsomal lipid peroxidation and as stimulators of microsomal NADPH oxidation. As noted above, menadione, 1,4-naphthoquinone, and 5-hydroxymenadione were from commercial sources; the other five derivatives were synthesized as follows: 2,3-dimethyl-1,4-naphthoquinone was prepared by chromic acid oxidation of 2,3-dimethylnaphthalene (17), 3-hydroxymenadione was prepared from menadione by H_2O_2/H_2SO_4 treatment as described by Fieser (18), 3-methoxy- and 5-methoxymenadione were prepared by silver oxide-catalyzed methylation of the corresponding hydroxy compounds (19), and pentadecylmenadione was prepared from menadione by dipalmitoyl peroxide alkylation (20). The dipalmitoyl peroxide was prepared from palmitoyl chloride and sodium peroxide as described previously (20). The synthetic yields ranged from 30 to 100% and were similar to those previously reported. The reaction products were confirmed by low-resolution mass spectroscopy.

Assays of microsomal lipid peroxidation. Microsomes were prepared from the livers of eight male Sprague-Dawley rats, 200-250 g. The rats were killed by exsanguination and the livers were excised immediately, perfused with 1.15% KCl, and homogenized with a Brinkman Polytron homogenizer. The microsomes were isolated by differential centrifugation (16), resuspended in 1.15% KCl to a protein concentration of 15 mg/ml, and stored in aliquots at −70°C prior to use. Under these storage conditions, the activity of the microsomal lipid peroxidation system remained unchanged for at least 3 months.

The incubation mixture for the assays of NADPH-dependent lipid peroxidation consisted of liver microsomes (0.5 mg protein/ml) suspended in 0.1 M Tris-HCl buffer, pH 7.4, containing 12.5 μM ferric pyrophosphate. The reaction was initiated by the addition of 25 μl of 50 mM NADPH to the 2.5 ml of incubation mixture preincubated for 1 min at 37°C. Aliquots (1.0 ml) were withdrawn at 0 and 20 min after the NADPH was added; the malondialdehyde content of these samples was measured by the thiobarbituric acid method (16, 21). The extent of lipid peroxidation was expressed as nanomoles malondialdehyde produced per 20 minutes per milligram microsomal protein.

The measurements of cumene hydroperoxide-dependent lipid peroxidation were performed similarly except that the ferric pyrophosphate was omitted and the reactions were initiated by the addition of 25 μl of 30 mM cumene hydroperoxide to the 2.5-ml incubation mixtures.

Antioxidant activity measurements. Acetone stock solutions of each of the eight naphthoquinones were prepared and were diluted serially. A graded series of at least six quinone concentrations were generated by adding 25 μl of each dilution to a series of 2.5-ml lipid peroxidation incubation mixtures, pH 7.4, unless otherwise indicated. The reactions were initiated by NADPH additions and the malondialdehyde accumulating in 20 min was measured and plotted as a function of quinone concentration. These plots were interpolated to obtain the IC_{50} values; i.e., the concentrations of quinone estimated to reduce the malondialdehyde accumulation by 50%.

Menadione was also tested as an antioxidant in the cumene hydroperoxide-stimulated system. Two series of incubation mixtures were prepared; in one series NADPH (0.5 mM) was included and in the other series it was omitted. Graded concentrations of menadione (0–5 μM final concentration) were added to the flasks in each series and, after preincubating at 37°C for 1 min, the flasks received cumene hydroperoxide to initiate peroxidation.

Assays of naphthoquinone-dependent NADPH oxidation. Each of the eight naphthoquinones was tested as a substrate for the microsomal menadione

reductase by measuring the rate of NADPH oxidation occurring in the presence of microsomes and the quinone (22). In each case, graded concentrations were chosen so that the Michaelis constants could be estimated. A Kontron Model 800 uv-vis recording spectrophotometer equipped with an automatic sample changer was used to obtain these measurements. The reference cuvette and the six sample cuvettes contained 2.5 ml of 0.1 M Tris-HCl buffer, pH 7.4 (unless otherwise indicated), 0.25 mM NADPH, and microsomes (0.5 mg protein/ml). With the monochromator set at 340 nm and the cuvettes warmed to 37°C NADPH oxidation in the sample cuvettes was initiated by adding 25 μl of a series of naphthoquinone stock solutions (in acetone) to the six sample cuvettes. The rates of NADPH oxidation were recorded for 5 min and the recordings were used to calculate the initial velocities, in nanomoles NADPH oxidized per minute per milligram microsomal protein. These data were used to estimate the apparent Michaelis constants (K_m, V_{max}) by the method of Wilkinson (23).

Other methods. To estimate the amount of lipid hydroperoxide that had accumulated in the microsomal membranes after 20 min of incubation with NADPH, ferric pyrophosphate, and a selected concentration of menadione, 4 ml of the incubation mixture described above was extracted with an equal volume of chloroform:methanol, 2:1 (24). The organic extract containing microsomal lipids was concentrated to dryness with a stream of N_2 and the residue was redissolved in 1.0 ml of methanol. An aliquot (100 μl) of the methanolic solution was added to a sample cuvette containing 2.5 ml of freshly prepared 1% KI in glacial acetic acid. The reference cuvette contained only the KI-acetic acid reagent. The increase in absorbance at 360 nm due to the reaction of the hydroperoxide with I^- (25) was recorded for 2 min; the hydroperoxide concentration was calculated from a standard curve prepared with graded concentrations of cumene hydroperoxide.

The apparent pK_a's of the hydroxylated menadione derivatives were estimated by recording the absorbance of the ionized forms as a function of pH. The anions of 3-hydroxymenadione and 5-hydroxymenadione were found to absorb maximally at 500 and 510 nm, respectively; the unionized forms showed negligible absorption at these wavelengths.

TABLE I

EFFECT OF MENADIONE ON NADPH + Fe^{2+}-CATALYZED MICROSOMAL LIPID PEROXIDATION

Menadione concentration (nM)	MDA accumulation (nmol 20 min^{-1} mg $protein^{-1}$)	Hydroperoxide accumulation (nmol 20 min^{-1} mg $protein^{-1}$)
0	84.6	15.0
25	65.4	16.5
50	0.76	1.87
100	0.62	1.00
150	0.65	1.50
200	0.70	0.50

Note. Results of a typical experiment are shown.

TABLE II

INHIBITION OF CUMENE HYDROPEROXIDE-DEPENDENT MICROSOMAL LIPID PEROXIDATION BY MENADIONE

Menadione concentration (μM)	Malondialdehyde accumulation (nmol 20 min^{-1} mg $protein^{-1}$)	
	−NADPH	+NADPH
0	16.2	9.2
0.2	15.8	5.8
0.5	15.4	2.5
1.0	15.7	1.6
2.0	15.0	1.0
5.0	15.6	1.5

Note. Results of a typical experiment are shown.

RESULTS

In a preliminary experiment, menadione was found to inhibit NADPH-dependent microsomal lipid peroxidation in a concentration-dependent manner. The concentration dependence was independent of incubation time for at least 20 min, after which no further malondialdehyde accumulation occurred. Table I shows that menadione, at concentrations of 50 nM or higher, effectively blocked NADPH-dependent microsomal lipid peroxidation, as measured either by the accumulation of malondialdehyde or of lipid hydroperoxides. It was also of interest to assess the antioxidant activity of menadione in a system where lipid peroxidation does not require NADPH. For this experiment, cumene hydroperoxide was chosen as the initiator of lipid peroxidation. The results (Table II) indicate that menadione (0.2–5.0 μM) is not an effective inhibitor of cumene hydroperoxide-dependent lipid peroxidation unless NADPH is included in the incubation mixture. These results also

TABLE III

STRUCTURE-ACTIVITY RELATIONSHIPS AMONG 1,4-NAPHTHOQUINONES

Structure			Activity		
				Stimulation of NADPH oxidation	
R_1	R_2	R_3	Inhibition of lipid peroxidation [IC_{50} (μM)]	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})
H	H	H	27	27.9 ± 6.0	9.1 ± 1.3
CH_3	H	H	0.037	34.1 ± 5.6	13.6 ± 1.3
CH_3	CH_3	H	0.130	20.5 ± 5.8	10.8 ± 2.1
CH_3	OH	H	180	57.0 ± 8.7	0.8 ± 0.1
CH_3	OCH_3	H	0.51	24.2 ± 9.5	10.5 ± 1.8
CH_3	$C_{15}H_{31}$	H	800	7.3 ± 2.8	0.9 ± 0.01
CH_3	H	OH	0.25	10.4 ± 0.3	12.9 ± 1.4
CH_3	H	OCH_3	0.47	11.8 ± 1.0	8.4 ± 0.3

Note. R_1 is at the 2 position; R_2 at the 3 position; and R_3 at the 5 position.

show that, in the absence of menadione, NADPH produces a partial inhibition of the cumene hydroperoxide-dependent lipid peroxidation, in agreement with the results of previous workers (26).

The observations noted above (Table II) suggested that the antioxidant activity of a given naphthoquinone may bear a relation to its activity as a substrate for microsomal quinone reductase. This possibility was examined by comparing eight 1,4-naphthoquinones as antioxidants and as stimulators of microsomal NADPH oxidation. As antioxidants, the quinones varied over a potency range of approximately 2×10^4. Although the two weakest antioxidants, 3-hydroxymenadione and 3-pentadecylmenadione, were also the two weakest quinone reductase substrates, a close quantitative relationship between inhibition of lipid peroxidation and stimulation of NADPH oxidation was not apparent: 1,4-naphthoquinone, menadione, 3-methylmenadione, 3-methoxymenadione, 5-hydroxymenadione, and 5-methoxymenadione were similar in activity as NADPH oxidase stimulators, but antioxidant activity among these compounds varied widely (Table III). Particularly striking was the difference in antioxidant potency between menadione (IC_{50} = 0.037 μM) and its desmethyl analog, 1,4-naphthoquinone (IC_{50} = 27 μM), despite being approximately equivalent in their ability to stimulate NADPH oxidation.

The structure-activity results revealed a striking difference in potency between the two hydroxylated compounds, 3-hydroxymenadione (IC_{50} = 180 μM) and 5-hydroxymenadione (IC_{50} = 0.25 μM), which was not apparent between the two corresponding methoxy compounds (Table III). These observations suggested that the difference in potency between the hydroxylated compounds may be largely explained by differences in their acid-base properties. An experimental determination of the dissociation constants yielded pK_a values of 9.1 and 5.1 for 5-hydroxy- and 3-hydroxymenadione, respectively, indicating that the latter compound is approximately 99.5% ionized at pH 7.4 (Fig. 1). This finding clearly suggested that the relative inactivity of 3-hydroxymenadione may have reflected its state of ionization at pH 7.4.

The antioxidant activity of 3-hydroxymenadione measured at pH 7.0 and 6.0 increased in proportion to the percentage increase in the undissociated form (Table IV).

Lowering the pH also improved the ability of this compound to stimulate NADPH oxidation. These results suggest

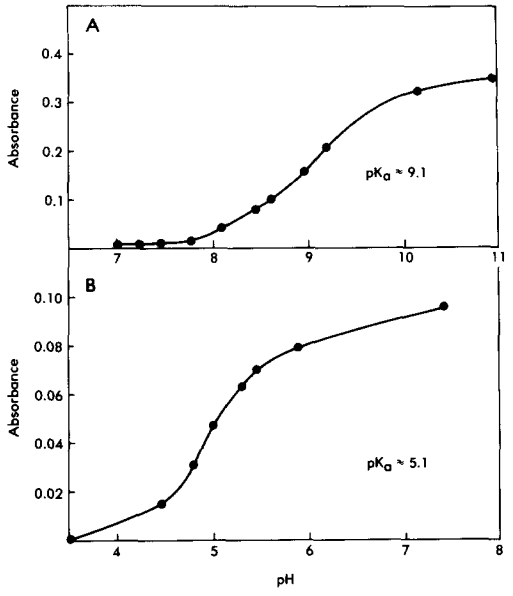

FIG. 1. pH titration curves for the anionic forms: (A) 5-hydroxymenadione; (B) 3-hydroxymenadione.

that the active antioxidant form of 3-hydroxymenadione is the reducible, undissociated form. At pH 7.4, 7.0, and 6.0 the IC_{50} value for this form appears to be nearly constant and approximately 0.9 μM, i.e., IC_{50} = (apparent IC_{50}) × (fraction undissociated species). Attempts to measure the antioxidant activity of 5-hydroxymenadione at pH's approaching its pK_a failed because at these pH's the microsomal lipid peroxidation system was inactivated.

DISCUSSION

The results presented in this paper provide new information on the mechanism and structure-activity relationships underlying the inhibition of microsomal lipid peroxidation by 1,4-naphthoquinones. In an earlier paper, Wills (27) suggested that menadione and related naphthoquinones may inhibit microsomal lipid peroxidation by more than one mechanism. Since reducing equivalents are consumed by microsomal quinone reduction and by microsomal lipid peroxidation, Wills (27) suggested that menadione may divert electrons that would otherwise catalyze lipid peroxidation. On the other hand, Wills (27) noted that menadione inhibited lipid peroxidation even when excess NADPH was present, and suggested that an antioxidant effect independent of electron diversion may contribute importantly to the inhibitory effect.

Several observations now indicate that electron diversion per se cannot account for the antioxidant activity of menadione. The menadione concentration required to inhibit microsomal NADPH-dependent lipid peroxidation is approximately three orders of magnitude below the concentration required to stimulate half-maximally the rate of NADPH oxidation. Moreover, 1,4-naphthoquinone and menadione are about equally effective in stimulating NADPH oxidation but menadione is about three orders of magnitude more potent as an antioxidant. The weakest antioxidant, 3-pentadecylmenadione, was ineffective at concentrations where a certain amount of stimulation of NADPH oxidation did occur. Finally, menadione, in the presence of NADPH, inhibited cumene hydroperoxide-dependent microsomal lipid peroxidation; in this model system NADPH inhibits rather than stimulates the reaction. Thus, it seems clear that the antioxidant action of menadione is unrelated to electron diversion, but is a result of a reaction between either menadione itself or one of its reduced forms with chemical species that are critically involved in the catalysis of microsomal lipid peroxidation.

It seems likely that the active antioxidant form of menadione is either the

TABLE IV

EFFECT OF pH ON 3-OH MENADIONE REDUCTION AND ON ITS ANTIOXIDANT ACTIVITY

pH	Percentage undissociated 3-OH-MD	Apparent antioxidant potency [IC_{50} (μM)]	NADPH oxidase stimulation (V_{max}/K_m)
7.4	0.50	180	0.014
7.0	1.05	75	0.028
6.0	10.50	9	0.078

semiquinone or hydroquinone, because of the demonstrated requirement for reducing equivalents (Table II). The antioxidant effect could therefore result from reactions between the hydroquinone and free radical species involved in catalyzing the propagation of lipid peroxidation. Studies with quinones in chemical systems (28) indicate that hydroperoxy radicals could be among those trapped. In the cumene hydroperoxide stimulated reaction, there is evidence from studies with reconstituted systems (7, 11) and with microsomes (29) that ferric cytochrome *P*-450 reacts with cumene hydroperoxide to form cumoxy radicals. Once again the hydroquinone could trap these radicals thereby preventing the initiation of lipid peroxidation. It is also possible that the semiquinone of menadione may contribute to the antioxidant activity by pairing with hydroperoxy or alkoxy radicals. The free electron in menadione semiquinone is delocalized so the semiquinone may be viewed as a composite of eight separate resonance forms. The structure-activity study presented here demonstrates the importance of a single methyl group on the quinone ring (cf. antioxidant activity of menadione vs that of 1,4-naphthoquinone), suggesting that the inductive properties of the methyl substituent may serve to stabilize forms that are important for optimal antioxidant activity (28). Also, the structure-activity study demonstrates the importance of an unhindered C-3 or C-5 site, suggesting that these sites may be importantly involved in the antioxidant reaction. Further studies with naphthoquinones that are substituted at other positions are needed to assess the importance of other sites on the naphthoquinone nucleus.

An interesting topic for future research is the question of whether the pro-oxidant and antioxidant properties of naphthoquinones can be uncoupled. Central to this question would be a comparison of the structure-activity relationships governing superoxide generation with those governing antioxidant activity. If different resonance forms are more important to one pathway than to the other, different structure-activity relationships may be expected. If this is the case, it should be possible to maximize or minimize one activity (pro-oxidant or antioxidant) in relation to the other by varying the positions of substitution or the nature of the substituents on the naphthoquinone nucleus. This capability could be important in the design of new pharmacologically active naphthoquinones.

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