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MECHANISMS OF TOXICITY OF NAPHTHOQUINONES TO ISOLATED HEPATOCYTES*

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Abstract—The possible mechanisms of naphthoquinone-induced toxicity to isolated hepatocytes were investigated using three structurally-related naphthoquinones, 1,4-naphthoquinone (1,4-NQ), 2-methyl-1,4-naphthoquinone (2-Me-1,4-NQ) and 2,3-dimethyl-1,4-naphthoquinone (2,3-diMe-1,4-NQ). 1,4-NQ was more toxic than 2-Me-1,4-NQ whereas 2,3-diMe-1,4-NQ did not cause cell death at the solubility-limited concentrations used. All three naphthoquinones extensively depleted intracellular glutathione (GSH). However, the depletion of GSH induced by 1,4-NQ and 2-Me-1,4-NQ prior to cell death was more rapid and extensive than that induced by the nontoxic 2,3-diMe-1,4-NQ. Further studies demonstrated that 2,3-diMe-1,4-NQ was cytotoxic in the presence of dicoumarol, a compound which also potentiates the cytotoxicity of 1,4-NQ and 2-Me-1,4-NQ. To investigate the differential cytotoxicity of these three naphthoquinones, their relative capacities to redox cycle and to bind covalently to cellular nucleophiles were assessed. Redox cycling was investigated using rat liver microsomes where the order of potency for quinone-stimulated redox cycling was 1,4-NQ ≈ 2-Me-1,4-NQ ⩾ 2,3-diMe-1,4-NQ as indicated by nonstoichiometric amounts of NADPH oxidation and O_2 consumption. NADPH-cytochrome P-450 reductase was implicated as the enzyme primarily responsible for naphthoquinone-stimulated redox cycling. The reactivity of the naphthoquinones with glutathione and, by implication, with other cellular nucleophiles was 1,4-NQ > 2-Me-1,4-NQ ⩾> 2,3-diMe-1,4-NQ. Overall, these studies indicate that 2,3-diMe-1,4-NQ is not cytotoxic (except in the presence of dicoumarol) and this lack of toxicity may be related either to its lesser capacity to redox cycle and/or its inability to react directly with cellular nucleophiles.

Quinones are widely distributed in nature, playing both an essential biological role in mitochondrial respiration and plant photosynthesis [1] and occurring as compounds of potential toxicological significance in foodstuffs and environmental pollutants [1, 2]. Several anti-cancer drugs contain the quinone nucleus [3] and adriamycin, a synthetic quinone, has proved useful in cancer chemotherapy [4], despite a dose-limiting cumulative cardiotoxicity [5]. It has been proposed that quinone cytotoxicity is related to "oxidative stress" arising from the capacity of these compounds to redox cycle [6–8].

Naphthoquinones can undergo metabolism either via a NAD(P)H-quinone oxidoreductase (DT-diaphorase)-catalysed two-electron reduction to a hydroquinone or a one-electron reduction catalysed by flavoenzymes such as NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase or NADH-ubiquinone reductase to form semiquinone radicals [9–12]. This radical is thought to initiate a redox cycle since it can readily autooxidize in the presence of dioxygen to regenerate the naphthoquinone and form the superoxide anion radical ($O_2^{\cdot-}$) [13, 14]. Alternatively, the semiquinone radical could undergo a second one-electron reduction to form the hydroquinone. Hydroquinone formation, either via DT-diaphorase or two one-electron reduction steps, has been considered as a detoxification process since the hydroquinone may be further metabolized to glucuronide and sulphate conjugates.

The cytotoxicity of naphthoquinones to isolated hepatocytes has been well documented [7, 11, 15]. Orrenius and coworkers [7, 16] have reported that metabolism of menadione (2-Me-1, 4-NQ) in isolated hepatocytes is associated with a depletion of reduced glutathione (GSH) and a concomitant formation of large amounts of oxidized glutathione (GSSG), suggesting dismutation of $O_2^{\cdot-}$ to H_2O_2 and the subsequent oxidation of GSH by GSH peroxidase [17]. These workers also observed that, prior to cell death, many small blebs appeared on the surface of menadione-treated hepatocytes [7]. The bleb formation was associated with altered intracellular thiol and Ca^{2+} homeostasis and it was proposed that these events were the result of cellular oxidative stress [7, 16, 18, 19].

In the present studies, we have investigated possible mechanisms by which three structurally related naphthoquinones, 1,4-naphthoquinone (1,4-NQ), 2-methyl-1,4-naphthoquinone (2-Me-1,4-NQ) and 2,3-dimethyl-1,4-naphthoquinone (2,3-diMe-1,4-NQ) (Fig. 1) could cause cell death. These compounds were chosen in order to ascertain the relative importance of oxidative stress and covalent binding in the mechanism(s) of cytotoxicity of the naphthoquinones. Both 1,4-NQ and 2-Me-1,4-NQ contain electrophilic centres at the 2 and/or 3 positions

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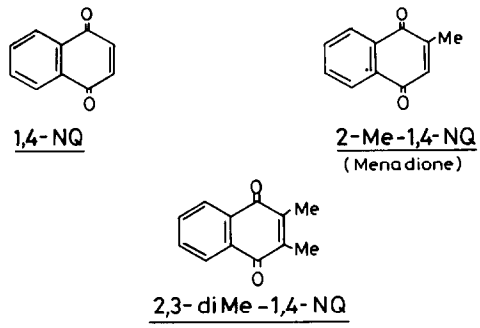

Fig. 1. Structures of naphthoquinones. 1,4-Naphthoquinone (1,4-NQ), 2-methyl-1,4-naphthoquinone (2-Me-1,4-NQ) and 2,3-dimethyl-1,4-naphthoquinone (2,3-diMe-1,4-NQ).

[20] and may bind to cellular nucleophiles. However, 2,3-diMe-1,4-NQ would be expected to exert its toxicity solely by redox cycling. Our results showed that 1,4-NQ and 2-Me-1,4-NQ were toxic to isolated hepatocytes and readily initiated redox cycling in microsomes as assessed both by NADPH oxidation and O_2 consumption. In contrast, 2,3-diMe-1,4-NQ was relatively nontoxic but was an order of magnitude less potent than both 1,4-NQ and 2-Me-1,4-NQ in its ability to redox cycle.

MATERIALS AND METHODS

Materials

Collagenase (from *Clostridium histolyticum*) was obtained from Boehringer-Mannheim GmbH, Mannheim, F.R.G. All other chemicals were purchased from Sigma Chemical Co., London, U.K., unless otherwise stated. 2-Me-1,4-NQ and 1,4-NQ were purchased from Fluka, Switzerland (99.8% purity) and the latter was purified by sublimation. 2,3-diMe-1,4-NQ was a gift from Dr. V. Narayanan, National Cancer Institute, Bethesda, U.S.A.

Animals

Male Sprague–Dawley rats (Olac Ltd, Shaw's Farm, Blackthorn, Bicester, U.K.) weighing 200–300 g were used in all experiments. The animals were allowed food and water *ad libitum*.

Hepatocyte isolation and incubation

Rat hepatocytes were prepared by collagenase perfusion of the liver [21]. The total yield of hepatocytes for each preparation ranged from 200 to 400×10^6 cells/liver. Initial viability (80–90%) and viability during the course of experiments were assessed by trypan blue exclusion. The cells (1×10^6 cells/ml) were incubated under a 95% oxygen:5% CO_2 atmosphere, in Krebs–Henseleit buffer, pH 7.4 containing 25 mM HEPES (bis-hydroxyethyl-1-piperazine ethane sulphonic acid) at 37° in rotating round bottom flasks [15]. The naphthoquinones, dissolved in dimethyl sulphoxide (DMSO), and dicoumarol, dissolved in alkalinized distilled water (pH 9.5), were added to the hepatocyte suspension in a 10 μl volume.

GSH determination

(a) *Isolated hepatocytes.* Aliquots of the cell suspension (0.5 ml) were taken throughout the time course of the incubation. The cell pellet and supernatant were separated by low speed centrifugation for 3 min and the supernatant discarded. Trichloroacetic acid (6.5%, 0.5 ml) was used to precipitate the protein in the cell pellet and hepatocellular levels of GSH, the major non-protein sulphydryl in the cell, were measured as acid-soluble thiols using the *o*-phthaldialdehyde assay method [22].

(b) *Buffered solutions.* The concentration of GSH in Krebs–Henseleit buffer, pH 7.4 containing 25 mM HEPES and incubated at 37° in the presence and absence of naphthoquinones, was measured by the *o*-phthaldialdehyde method [22]. After addition of naphthoquinones dissolved in DMSO, the DMSO concentration of the incubate was 1% (v/v).

Preparation of rat liver microsomes

Washed liver microsomes were prepared according to the method of Ernster *et al.* [23]. The pellet was resuspended in 100 mM Tris buffer pH 7.5 containing 50 mM KCl. Microsomal protein was determined by the method of Lowry *et al.* [24] using bovine serum albumin as a standard. Freshly prepared microsomes were used in all experiments.

Partial purification of DT-diaphorase

DT-diaphorase was partially purified from the livers of male Sprague–Dawley rats by the method of Ernster *et al.* [9, 25]. DT-diaphorase activity was assayed using a modified method of Ernster *et al.* [9, 25] with menadione (2-Me-1,4-NQ) as substrate. The cuvettes contained 100 mM sodium phosphate buffer pH 7.4, 250 μM NADPH, 5 μM FAD, bovine serum albumin (250 μg) and 40 μM menadione in a final volume of 1 ml at 25°. DT-diaphorase activity was measured as dicoumarol-inhibitable NADPH oxidation.

NADPH oxidation by rat liver microsomes

NADPH oxidation was followed spectrophotometrically at 340 nm, using an extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$. The reaction mixture contained, in a final volume of 1 ml, 150 μM NADPH and 0.4 mg microsomal protein in a 100 mM Tris, 50 mM KCl buffer, pH 7.5. Incubations were carried out at 37°.

Oxygen consumption by rat liver microsomes

Oxygen consumption was measured polarographically using a water-jacketed cell fitted with a Clarke oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) as previously described [14]. Incubations were carried out at 37° with a reaction mixture which contained, in a final volume of 2.5 ml, 150 μM NADPH and 1 mg microsomal protein suspended in a buffer of 100 mM Tris, 50 mM KCl, pH 7.5.

RESULTS

Toxicity of 1,4-*NQ,* 2-*Me*-1,4-*NQ and* 2,3-*diMe*-1,4-*NQ to isolated hepatocytes*

Isolated hepatocytes were most susceptible to the toxic effects of 1,4-NQ, cell death occurring at con-

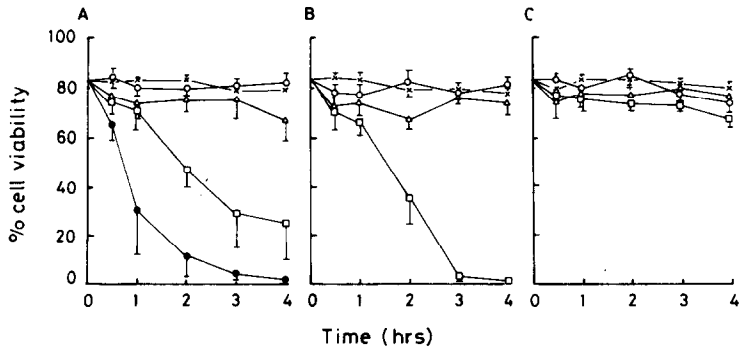

Fig. 2. Time course of toxicity of naphthoquinones to isolated rat hepatocytes. Hepatocytes were incubated in the absence (×—×) or presence of various concentrations of (A) 1,4-NQ, 20 μM (○—○), 50 μM (△—△), 75 μM (□—□) or 100 μM (●—●); (B) 2-Me-1,4-NQ, 50 μM (○—○), 100 μM (△—△), or 200 μM (□—□) and (C) 2,3-diMe-1,4-NQ, 100 μM (○—○), 200 μM (△—△) or 300 μM(□—□). Cell viability was assessed by trypan blue exclusion. Values are means ± S.E.M. obtained from three separate experiments.

centrations of 75 and 100 μM (Fig. 2A). 2-Me-1,4-NQ was less toxic than 1,4-NQ with cell death becoming apparent at a concentration of 200 μM (Fig. 2B). In contrast, 2,3-DiMe-1,4-NQ caused no loss in cell viability up to concentrations of 300 μM (Fig. 2C) above which crystals began to form in the incubation media. However, in the presence of dicoumarol, a DT-diaphorase inhibitor (9,25), 2,3-diMe-1,4-NQ caused 100% loss in cell viability after 3 hr incubation (Table 1). Dicoumarol is also known to potentiate the cytotoxicity of both 1,4-NQ [15] and 2-Me-1,4-NQ [7]. Further analysis of these results was carried out by plotting the log naphthoquinone concentration versus % cell death after a 4 hr incubation, thus allowing calculation of the LC_{50}, i.e. the naphthoquinone concentration which caused 50% cell death (figure not shown). The LC_{50}s for 1,4-NQ, 2-Me-1,4-NQ and 2,3-diMe-1,4-NQ were 65, 130 and ⩾300 μM respectively. These data demonstrated that the unsubstituted 1,4-NQ was more toxic than the monosubstituted 2-Me-1,4-NQ and that the disubstituted 2-3-diMe-1,4-NQ was nontoxic at the solubility-limited concentrations used in this study.

Table 1. Effect of dicoumarol on the toxicity of 2,3-diMe-1,4-NQ to isolated hepatocytes

Conditions	% Cell viability Incubation time (hr) 0	0.5	1	2	3	4
Control	84	81	79	75	69	70
Dicoumarol		76	84	75	69	70
2,3-diMe-1,4-NQ		75	83	80	75	72
2,3-diMe-1,4-NQ plus dicoumarol		67	63	39	0	—

% Cell viability was assessed in hepatocytes incubated with dicoumarol (20 μM) alone and with 2,3-diMe-1,4-NQ (300 μM) in the absence and presence of dicoumarol (20 μM). Values are representative of results obtained from three separate experiments.

Effect of naphthoquinones on intracellular levels of reduced glutathione in isolated hepatocytes

The addition of either 1,4-NQ, 2-Me-1,4-NQ or 2,3-diMe-1,4-NQ to hepatocytes caused a concentration-dependent depletion of intracellular levels of reduced glutathione (Fig. 3). Moreover, lower concentrations of 1,4-NQ and 2-Me-1,4-NQ than of 2,3-diMe-1,4-NQ were required to decrease cellular glutathione levels. Of particular interest was the rapid loss of reduced glutathione, which occurred within 30 min after addition of 1,4-NQ and 2-Me-1,4-NQ to the incubation media (Fig. 3A and B). This rapid fall in glutathione levels was not noted in the presence of 2,3-diMe-1,4-NQ (Fig. 3C), where the concentration of glutathione fell more slowly over a longer time period. From these data, it was possible that the cellular events underlying or associated with the initial rapid depletion of hepatocellular levels of reduced glutathione were related to naphthoquinone-induced cell death.

Naphthoquinone-dependent NADPH utilization by rat liver microsomes

The relative abilities of the three naphthoquinones to redox cycle was assessed by measuring NADPH oxidation by rat liver microsomes in the presence of various concentrations of the naphthoquinones. The data indicated that 1,4-NQ and 2-Me-1,4-NQ were an order of magnitude more potent than 2,3-diMe-1,4-NQ in causing an increase in microsomal NADPH oxidation (Fig. 4). These results also demonstrated the ability of naphthoquinones to redox cycle since the amount of naphthoquinones added to the microsomal preparation was substantially less than the number of nmoles of NADPH utilized, e.g. for the 1 μM concentration of 2-Me-1,4-NQ, 2.5 nmoles of naphthoquinone were added to 1 mg of microsomal protein and 100 nmoles of NADPH were oxidized. Non-stoichiometric amounts of NADPH were utilized in the presence of all three quinones at all the concentrations used except 100 μM 2,3-diMe-1,4-NQ, where the amount of quinone added equalled the amount of NADPH utilized.

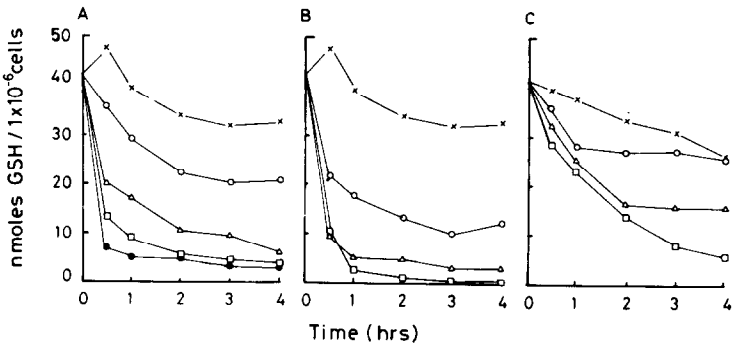

Fig. 3. Time course of naphthoquinone-dependent glutathione depletion in isolated rat hepatocytes. Hepatocytes were incubated in the absence (×—×) or presence of various concentrations of (A) 1,4-NQ, 20 μM (○—○), 50 μM (△—△), 75 μM (□—□) or 100 μM (●—●); (B) 2-Me-1,4-NQ, 50 μM (○—○), 100 μM (△—△) or 200 μM (□—□) and (C) 2,3-diMe-1,4-NQ, 100 μM (○—○), 200 μM (△—△) or 300 μM (□—□). Hepatocellular glutathione levels were measured as described in Materials and Methods throughout the 4 hr incubation. Values are representative of results obtained from three separate cell preparations.

Naphthoquinone-dependent oxygen consumption by rat liver microsomes

In order to assess further the capacity of naphthoquinones to redox cycle, naphthoquinone-dependent microsomal O_2 consumption was measured. In agreement with the results obtained for NADPH utilization, non-stoichiometric levels of O_2 consumption were observed and 1,4-NQ and 2-Me-1,4-NQ were an order of magnitude more potent than 2,3-diMe-1,4-NQ in their ability to stimulate microsomal O_2 consumption (Fig. 5). In combination, these data indicate that the relative abilities of the naphthoquinones to redox cycle were 1,4-NQ ≃ 2-Me-1,4-NQ ⩾ 2,3-diMe-1,4-NQ.

Modulation of naphthoquinone-dependent NADPH utilization and O_2 consumption by rat liver microsomes

The results illustrated in Tables 2 and 3 generally demonstrated parallel changes in NADPH utilization and O_2 consumption. NADPH was the preferred cofactor since only a negligible amount of NADH was utilized in the presence of naphthoquinones (Table 2). Addition of $NADP^+$ (2 mM), an inhibitor of NADPH-cytochrome P-450 reductase [12], partially inhibited naphthoquinone-stimulated redox cycling (Tables 2 and 3). Furthermore, dicoumarol,

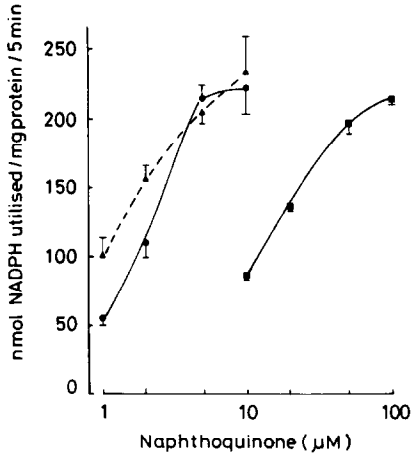

Fig. 4. Naphthoquinone-dependent stimulation of microsomal NADPH oxidation. Rat liver microsomes treated with 1,4-NQ (1–10 μM) (●—●), 2-Me-1,4-NQ (1–10 μM) (▲—▲), and 2,3-diMe-1,4-NQ (10–100 μM) (■—■). NADPH disappearance was measured as described in Materials and Methods. Values are means ± S.E.M. obtained from at least three separate microsomal preparations and were corrected for a basal rate of 65.5 nmoles NADPH oxidized/mg protein/5 min.

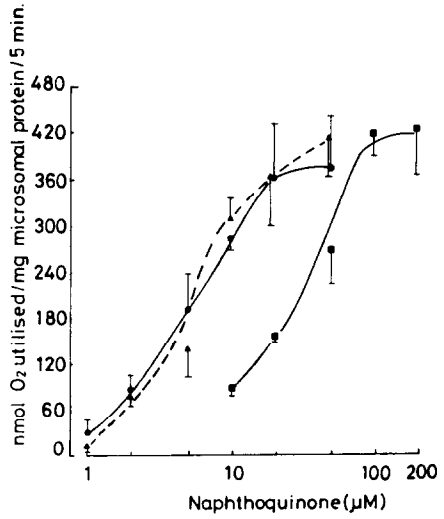

Fig. 5. Naphthoquinone-dependent stimulation of microsomal O_2 consumption. Rat liver microsomes were treated with 1,4-NQ (1–60 μM) (●—●), 2-Me-1,4-NQ (1–60 μM) (▲—▲) and 2,3-diMe-1,4-NQ (10–200 μM) (■—■). O_2 consumption was measured as described in Materials and Methods. Values are means ± S.E.M. obtained from at least three separate microsomal preparations and were corrected for a basal rate of 50 nmoles O_2 consumed/mg protein/5 min.

Table 2. Naphthoquinone-stimulated NADPH oxidation by rat liver microsomes

Conditions	NAD(P)H oxidation (nmoles/5 min/mg microsomal protein)		
	1,4-NQ (5 μM)	2-Me-1,4-NQ (5 μM)	2,3-diMe-1,4-NQ (20 μM)
Microsomes:			
NADPH (150 μM)	176.5 ± 5.1	173.8 ± 7.9	147.8 ± 5.9
Plus $NADP^+$ (2 mM)	73.8 ± 3.1*	58.3 ± 2.4*	39.3 ± 1.1*
Dicoumarol (30 μM)	212 ± 3.9*	211.5 ± 8.7*	153.8 ± 13.1
Partially purified DT-diaphorase (0.2 U)	44.6 ± 1.8*	83.5 ± 7.1*	173.1 ± 11.3
Partially purified DT-diaphorase (0.2 U) and Dicoumarol (30 μM)	163.5 ± 2.0	164.5 ± 5.3	130.3 ± 5.7
Glutathione (50 μM)	122.0 ± 7.7*	166.2 ± 2.4	146.0 ± 6.1
NADH (150 μM)	14.1 ± 8.7	8.7 ± 5.8	4.4 ± 3.6
Microsomes + cytosol:			
NADPH (150 μM)	0.5 ± 0.2*	65.3 ± 2.2*	219.0 ± 8.9*
Plus Dicoumarol (30 μM)	23.3 ± 2.9†	184.5 ± 6.7†	159.3 ± 8.1†

The assays were performed as described in Materials and Methods. Values are means ± S.E.M. obtained from six (microsomes plus NADPH alone) or three (all other conditions) separate microsomal preparations and were corrected for a basal rate of 65.5 nmoles NADPH oxidized/mg protein/5 min.

* Significantly different from control incubations of microsomes plus NADPH, $P < 0.05$.

† Significantly different from control incubations of microsomes + cytosol plus NADPH, $P < 0.05$.

an inhibitor of DT-diaphorase [9, 12, 25], both at the 30 μM concentration illustrated here and at 100 μM (results not shown), slightly increased NADPH oxidation in the presence of 1,4-NQ and 2-Me-1,4-NQ but not 2,3-diMe-1,4-NQ, yet did not significantly affect O_2 disappearance from the microsomal incubation. In contrast, addition of DT-diaphorase (0.2 U) markedly inhibited the NADPH oxidation catalysed by 1,4-NQ and 2-Me-1,4-NQ yet modestly increased NADPH oxidation caused by 2,3-diMe-1,4-NQ (Table 2). These effects were reversed by dicoumarol.

We also wished to assess the effects of cytosol and in particular cytosolic GSH on naphthoquinone-stimulated NADPH oxidation. Hence the concentration of GSH used in these studies (50 μM) was approximately the final concentration achieved after addition of 0.6 mg cytosolic protein to the 1 ml microsomal incubation mixture, (40.2 μM ± 8.4, mean ± S.E.M.). Glutathione (50 μM) decreased 1,4-NQ-dependent NADPH oxidation but had no effect on either 2-Me-1,4-NQ- or 2,3-diMe-1,4-NQ-catalysed oxidation of NADPH. Addition of cytosol (0.6 mg) almost completely abolished the response to 1,4-NQ, markedly decreased the response to 2-Me-1,4-NQ but in the presence of 2,3-diMe-1,4-NQ, modestly stimulated NADPH utilization yet had no significant effect on O_2 consumption (Tables 2 and 3). Dicoumarol completely reversed the inhibitory effects of cytosol on 2-Me-1,4-NQ-stimulated NADPH oxidation (Table 2) and partially reversed its effects on O_2 consumption (Table 3). Similarly, dicoumarol increased 1,4-NQ-stimulated NADPH utilization (Table 2) and O_2 consumption (Table 3). However, this effect was very modest and did not approach the microsomal level of redox cycling seen in the absence of cytosol. Conversely, for 2,3-diMe-1,4-NQ-stimulated NADPH utilization, which was enhanced by the addition of cytosol, dicoumarol again reversed the effects of cytosol (Table 2) but decreased O_2 consumption (Table 3).

Reaction of naphthoquinones with GSH in solution

In order to investigate the capacity of 1,4-NQ, 2-Me-1,4-NQ and 2,3-diMe-1,4-NQ to react directly with cellular nucleophiles, the effect of the naph-

Table 3. Naphthoquinone-stimulated O_2 consumption by rat liver microsomes

	O_2 consumption (nmoles/5 min/mg microsomal protein)		
	1,4-NQ (10 μM)	2-Me-1,4-NQ (10 μM)	2,3-diMe-1,4-NQ (50 μM)
Microsomes:			
NADPH (150 μM)	282 ± 53.7	309 ± 40.3	268 ± 46.1
Plus $NADP^+$ (2 mM)	134 ± 8.3*	180 ± 9.1*	163 ± 4.1*
Dicoumarol (30 μM)	246 ± 9.3	308 ± 17.6	271 ± 23.6
Microsomes + cytosol			
NADPH (150 μM)	8 ± 6.4*	84 ± 18.2*	241 ± 36.6
Plus Dicoumarol (30 μM)	30 ± 15.9	203 ± 6.2†	156 ± 6.4

The assays were performed as described in Materials and Methods. Values are means ± S.E.M. obtained from three separate microsomal preparations and were corrected for a basal rate of 50 nmoles O_2 consumed/mg protein/5 min.

* Significantly different from control incubations of microsomes plus NADPH, $P < 0.05$.

† Significantly different from control incubations of microsomes + cytosol plus NADPH, $P < 0.05$.

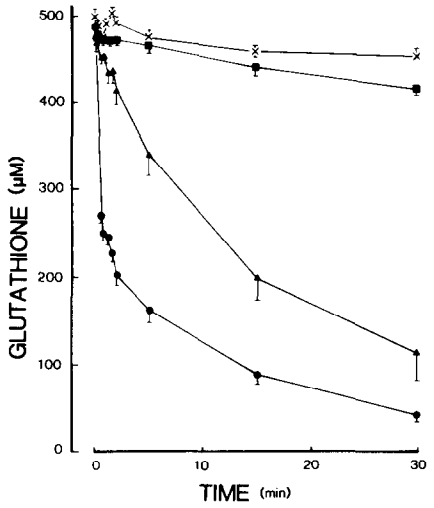

Fig. 6. Reaction of naphthoquinones with glutathione (GSH). Krebs–Henseleit buffer, pH 7.4, containing 25 mM HEPES and 500 μM GSH was incubated in the absence (×—×) and presence of 1,4-NQ (200 μM) (●—●), 2-Me-1,4-NQ (200 μM) (▲—▲) or 2,3-diMe-1,4-NQ (200 μM) (■—■). GSH levels were determined as described in Materials and Methods. Values are means ± S.E.M. obtained from three separate experiments.

thoquinones on the rate and extent of GSH disappearance from solution was examined (Fig. 6). 1,4-NQ (200 μM) reacted rapidly with GSH (500 μM) reducing the GSH concentration to less than 50% of control values within 20 sec. Following the initial rapid decline, a slower decrease occurred until nearly all of the GSH had been removed at the end of the 30 min incubation. 2-Me-1,4-NQ (200 μM) also reacted with GSH (Fig. 6) but much more slowly than 1,4-NQ. A moderate and consistent decrease in GSH concentration from 500 to 100 μM occurred over 30 min. The disappearance of GSH in the presence of both 1,4-NQ and 2-Me-1,4-NQ was greater than the maximal depletion expected from direct conjugation with GSH. In the case of 2-Me-1,4-NQ this has been accounted for by oxidation of GSH to GSSG (D. Ross, personal communication). In contrast, the levels of GSH in the presence of 2,3-diMe-1,4-NQ, both at the 200 μM concentration (Fig. 6) and at 300 μM (results not shown) were not significantly different from the levels in the control incubation.

DISCUSSION

The present studies have examined the cytotoxicity to isolated hepatocytes of three structurally related naphthoquinones. The mechanism by which quinonoid compounds cause cell death has been extensively studied and it has been postulated that toxicity is the result of "oxidative stress" arising from the capacity of these compounds to redox cycle [6–8]. However, the covalent reaction of quinones with cellular nucleophiles, e.g. sulphydryl-containing proteins, may also be important in cell death [16, 18]. As illustrated by the structures in Fig. 1, the three naphthoquinones used in this study would be predicted to have different covalent binding capacities. Hence, we have investigated the relationship between the cytotoxicity of these naphthoquinones and their capacity to redox cycle and/or to bind covalently to cellular nucleophiles.

In contrast to 1,4-NQ and 2-Me-1,4-NQ, 2,3-diMe-1,4-NQ was not toxic to isolated hepatocytes (Fig. 2). Similarly, 2,3-diMe-1-NQ was markedly less effective than 1,4-NQ and 2-Me-1,4-NQ in its capacity to redox cycle (Figs 4 and 5). However, all three naphthoquinones depleted hepatocellular GSH (Fig. 3). Since GSH depletion by 2,3-diMe-1,4-NQ was slow and maximal at 4 hr, it was possible that 2,3-diMe-1,4-NQ-treated cells would die subsequent to GSH depletion. However, cell death in the presence of 2,3-diMe-1,4-NQ was not a late event, since cell viability of 2,3-diMe-1,4-NQ treated hepatocytes was not different from control hepatocytes even when the incubation time was prolonged to 6 hr (data not shown). These data suggest that maximal depletion of glutathione was not necessarily associated with cytotoxicity and indicate that glutathione depletion alone was insufficient to cause cell death. The results also support the hypothesis that glutathione plays only a protective role in naphthoquinone cytotoxicity. Orrenius and coworkers have proposed that depletion of protein thiols, subsequent to glutathione depletion, is critically important in naphthoquinone-induced cytotoxicity [16, 18].

Naphthoquinone-dependent depletion of hepatocellular glutathione may be due to various mechanisms including (i) direct conjugation [20], (ii) redox cycling generating active oxygen species such as hydrogen peroxide, which can be detoxified by glutathione peroxidase, with concomitant formation of oxidized glutathione [17] and (iii) glutathione could participate in one-electron reduction of the naphthoquinones or the semiquinone radical with subsequent formation of the semiquinone or hydroquinone and the glutathionyl radical, which in turn may dimerise to form oxidized glutathione [26]. These mechanisms have been investigated by Orrenius *et al.* using menadione (2-Me-1,4-NQ). Hepatocellular glutathione depletion was largely accounted for by oxidation [7, 16], although a menadione–glutathione conjugate was also detected and represented approximately 15% of the total hepatocellular disappearance of glutathione [16].

In the present studies, it would be predicted that 1,4-NQ and 2-Me-1,4-NQ but not 2,3-diMe-1,4-NQ could deplete hepatocellular glutathione by direct conjugation. These structural considerations are in agreement with the chemical reactivity of these naphthoquinones with glutathione (Fig. 6). The formation of glutathione conjugates resulting from the direct interaction of 1,4-NQ or 2-Me-1,4-NQ with glutathione has been described previously [20]. However, as noted here, the disappearance of GSH from a solution containing 1,4-NQ or 2-Me-1,4-NQ was greater than could be accounted for by direct conjugation. This is in agreement with the observations of other workers who have measured formation of oxidized glutathione in the presence of naphthoquinones (D. Ross, personal communi-

cation). Furthermore, it has been reported that the menadione–glutathione conjugate itself can redox cycle [27]; therefore glutathione conjugation may not be a detoxification process. However, since the menadione–glutathione conjugate is rapidly transported out of the cell [16], glutathione conjugation could attenuate further redox cycling in the intact cell.

The capacity of the three naphthoquinones to redox cycle and the enzymes responsible for the one- and two-electron reduction of the naphthoquinones were studied using liver microsomes. The differential capacity to redox cycle (1,4-NQ ≈ 2-Me-1,4-NQ ≫ 2,3-diMe-1,4-NQ) could have been predicted from the work of Powis and Appel [28] who have proposed that the quinone one-electron reduction potential allows prediction of the flavoenzyme(s) involved in quinone one-electron reduction. For the three naphthoquinones used in the present study, the one-electron reduction potentials (E_7^1) were measured by pulse radiolysis and found to be −143, −203 and −296 mV for 1,4-NQ, 2-Me-1,4-NQ and 2,3-diMe-1,4-NQ respectively (I. Wilson and P. Wardman, personal communication). Based on the data of Powis and Appel [28], we would predict that the high redox potential of 2,3-diMe-1,4-NQ should make it a relatively poor substrate for NADPH-cytochrome P-450 reductase whereas both 1,4-NQ and 2-Me-1,4-NQ should be good substrates for this enzyme. In contrast, none of the quinones would be expected to be good substrates for NADH-cytochrome b_5 reductase. Our results were in good agreement with these predictions, in that NADH could not substitute for NADPH to support naphthoquinone-dependent redox cycling and $NADP^+$, an inhibitor of NADPH-cytochrome P-450 reductase inhibited [12], the redox cycling of all three naphthoquinones.

Further studies examined the involvement of cytosolic components in the metabolism of naphthoquinones (Tables 2 and 3). Redox cycling mediated by 2-Me-1,4-NQ was markedly inhibited both by the presence of cytosol and of partially purified DT-diaphorase. This inhibition was almost completely reversed by dicoumarol (Table 2). Hence the results are consistent with the two electron reduction of 2-Me-1,4-NQ by cytosolic DT-diaphorase decreasing the effective concentration of 2-Me-1,4-NQ available to redox cycle and are in agreement with other studies suggesting a protective role for DT-diaphorase against the toxicity of menadione [7, 12]. Addition of GSH had no effect on 2-Me-1,4-NQ-dependent redox cycling indicating that either the menadione-GSH conjugate also has the capacity to redox cycle or that the reaction of 2-Me-1,4-NQ with GSH was relatively slow (Fig. 6). In the case of 1,4-NQ, cytosol and DT-diaphorase as well as GSH effectively inhibited redox cycling (Table 2). However, although dicoumarol completely reversed the inhibition in the presence of DT-diaphorase it had only a slight effect on the inhibition which occurred in the presence of cytosol (Table 2). Thus with 1,4-NQ, it would appear that metabolism via cytosolic DT-diaphorase is less important than the interaction of 1,4-NQ with cytosolic components such as glutathione (Fig. 6) or other nucleophiles. In contrast to 1,4-NQ and 2-Me-1,4-NQ, addition of cytosol and DT-diaphorase to 2,3-diMe-1,4-NQ-treated microsomes caused an increase in NADPH oxidation which was reversed by dicoumarol. These results are more difficult to interpret but suggest that 2,3-diMe-1,4-NQ has a similar affinity for the enzymes catalysing both one- and two-electron reductive metabolism. Of further interest, in isolated hepatocyte studies, dicoumarol markedly potentiated both glutathione depletion and cytotoxicity in the presence of 2,3-diMe-1,4-NQ (Table 1), 1,4-NQ [15] and 2-Me-1,4-NQ [7]. This potentiation of cytotoxicity has been proposed to result from inhibition of the DT-diaphorase detoxification pathway, thus allowing more quinone to be metabolized via the one-electron toxic pathway to form reactive semiquinones and/or active oxygen species. For 1,4-NQ and 2-Me-1,4-NQ, the present data is largely compatible with this mechanism. However, other possibilities such as a synergistic toxicity of dicoumarol and quinones to mitochondria [29] cannot be excluded since DT-diaphorase-catalysed metabolism of 2,3-diMe-1,4-NQ (Table 2) was not extensive, yet dicoumarol markedly potentiated 2,3-diMe-1,4-NQ-dependent cytotoxicity.

In conclusion, a differential susceptibility to the cytotoxic effects of 1,4-NQ, 2-Me-1, 4-NQ and 2,3-diMe-1,4-NQ was observed in isolated hepatocytes. 1,4-NQ was the most potent cytotoxin and 2,3-diMe-1,4-NQ was nontoxic. A differential ability of the naphthoquinones to redox cycle in microsomal preparations was observed, with 1,4-NQ and 2-Me-1,4-NQ being much more active than 2,3-diMe-1,4-NQ. However, since the nontoxic 2,3-diMe-1,4-NQ has both a lesser capacity to redox cycle and does not directly covalently bind to cellular nucleophiles, our results cannot clearly differentiate between covalent binding and redox cycling as the primary mechanism of naphthoquinone-induced cytotoxicity. Furthermore, these studies indicate that depletion of intracellular glutathione may be essential but by itself is insufficient to cause toxicity and that NADPH-cytochrome P-450 reductase is the major enzyme involved in the one-electron reduction and redox cycling of the naphthoquinones catalysed by rat liver microsomes.

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