The Role of Oxidative Processes in the Cytotoxicity of Substituted 1,4-Naphthoquinones in Isolated Hepatocytes

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In order to clarify the role of oxidative processes in cytotoxicity we have studied the metabolism and toxicity of 2-methyl-1,4-naphthoquinone (menadione) and its 2,3 dimethyl (DMNQ) and 2,3 diethyl (DENQ) analogs in isolated rat hepatocytes. The two analogs, unlike menadione, cannot alkylate nucleophiles directly and were considerably less toxic than menadione. This decreased toxicity was consistent with the inability of DMNQ and DENQ to alkylate but we also found them to undergo lower rates of redox cycling in hepatocytes and a higher ratio of two electron as opposed to one electron reduction relative to menadione. Thus, facile analysis of the respective roles of alkylation and oxidation in cytotoxicity was not possible using these compounds. In hepatocytes pretreated with bischloroethyl-nitrosourea (BCNU) to inhibit glutathione reductase, all three naphthoquinones caused a potentiation of reduced glutathione (GSH) removal/oxidized glutathione (GSSG) generation and cytotoxicity relative to that observed in control cells. These data show that inhibition of hepatocyte glutathione reductase by BCNU results in enhanced naphthoquinone-induced oxidative challenge and subsequent cellular toxicity. That DMNQ and DENQ are cytotoxic, albeit at high concentrations, and that this cytotoxicity is potentiated by BCNU pretreatment suggest that oxidative processes alone can be a determinant of cytotoxicity.

The metabolism and toxicity of menadione, 2-methyl-1,4-naphthoquinone, have been extensively studied in isolated hepatocytes (1–5) which constitutes a useful model system for investigations of mechanisms of hepatotoxicity in vitro. Menadione-induced toxicity in hepatocytes is thought to involve depletion of cellular thiols such as glutathione (GSH), and thiol groups contained in proteins (4, 5). Recent evidence has implicated removal of cellular thiol groups to be the mechanism whereby menadione-induced perturbations in intracellular calcium ion homeostasis may occur (5, 6). These changes may lead to increases in cytosolic free calcium concentration, cytoskeletal alterations, and subsequent cell death (1, 2).

The removal of cellular thiols in cells by menadione is a consequence of two major
processes, alkylation and oxidation. Menadione is an electrophile and as such can react with nucleophiles directly, generating a menadione-GSH conjugate and alkylated protein in the case of GSH and protein SH groups, respectively (4, 7). Menadione is also a substrate for various one-electron reductases in both the mitochondrial and endoplasmic reticular compartments of the cell (1). These enzymes catalyze the production of semiquinone radicals derived from menadione whose subsequent reoxidation by molecular oxygen generates the parent quinone and superoxide anion radical (O₂⁻) (1). Dismutation of O₂ produces hydrogen peroxide (H₂O₂), and further reactions of these species can lead to the production of hydroxyl radical (OH) and singlet oxygen (¹O₂) (7, 8). Thus, an assortment of oxidizing species can be produced in the cell as a consequence of one-electron reduction of menadione, and such species will readily oxidize reduced thiols.

In an attempt to clarify the role of the generation of oxidizing species in quinone-induced cytotoxicity, we have studied the metabolism of menadione and two of its analogs which cannot alkylate nucleophilic sites directly. In this study we have compared the metabolism and toxicity of menadione, 2,3-dimethyl-1,4-naphthoquinone (DMNQ) and 2,3-diethyl-1,4-naphthoquinone (DENQ) to both control and BCNU pretreated hepatocytes and measured the effects of the quinones on cellular thiol homeostasis.

MATERIALS AND METHODS

1,4-Naphthoquinone was purchased from Aldrich. GSH, GSSG, dicoumarol, and menadione were obtained from Sigma, while collagenase (grade II), NADPH, and superoxide dismutase (SOD) were purchased from Boehringer-Mannheim. All other chemicals were of at least reagent grade and were purchased locally.

2,3-Dimethyl-1,4-naphthoquinone (DMNQ) and 2,3-diethyl-1,4-naphthoquinone (DENQ) were prepared as follows: a mixture of 1,4-naphthoquinone (1.58 g), propanoic acid (6.0 g), sulpholane (20 ml), water (25 ml), and silver nitrate (0.5 g) was heated to 80°C. Potassium peroxydisulfate (5.5 g) in water (25 ml) was added during 20 min at 80°C. After a further 15 min at this temperature, the mixture was poured onto ice (100 g). The water was decanted off and the residue dissolved in acetonitrile (50 ml), filtered, and evaporated to dryness. The residue was chromatographed on Silica gel, with petroleum ether (BP 60-80°C)/ethyl acetate (9/1) giving 2,3-diethyl-1,4-naphthoquinone (1.61 g; 75%; MPt 67-68°C, lit. MPt 68.5-69.5°C (10)). An analogous preparation was used for 2,3-dimethyl-1,4-naphthoquinone, using 1,4-naphthoquinone and acetic acid, in 79% yield (MPt 124-125°C; lit. MPt 125-126°C (11)). TLC was used to check the purity of these compounds (Silica plates; petroleum ether/ethyl acetate 3/1) DMNQ-one spot Rₜ = 0.85; DENQ-one spot Rₜ = 0.90. NMR analysis was totally consistent with the proposed structures.

Male Sprague–Dawley rats (200–250 g) were used in all experiments. Hepatocytes were isolated as in (12) and cell incubations were performed in rotating round-bottomed flasks (10⁶ cells/ml) under an atmosphere of 95% O₂ 5% CO₂. Glutathione reductase was inhibited by treatment of the cells with 1,3-bis-(2-chloroethyl)-1-nitrosourea essentially as previously described (13), except that Eagles minimal essential medium supplemented with 1 mM methionine was used for cell treatment and cell recovery. This treatment produces at least 90% inhibition of GSSG reductase without affecting cellular GSH content (13) and this was verified in these studies. Incubations were performed in Krebs–Henseleit buffer, pH 7.4, containing 12.5 mM Hepes at 37°C, and substrates were added in dimethylsulfoxide (0.4% of final volume except for toxicity curves of DMNQ and DENQ 0.6 mM (0.8%) and 1 mM (2%)). Control experiments showed that concentrations of up to 2% DMSO were not cytotoxic and had no effect on menadione-induced toxicity. Cell viability was determined by trypan blue exclusion. GSH and GSSG were determined by HPLC after de-
Rivatization as described by Reed et al. (14). \( \cdot \text{O}_2^- \) was detected by SOD-sensitive reduction of acetylated cytochrome c, prepared as in (15) using the wavelength pair 550–540 nm (16). Dialyzed whole liver homogenate was prepared by homogenization of the liver in 0.25 M sucrose (20% w/v), centrifugation at 2000 g for 10 min, followed by overnight dialysis of the supernatant against 0.15 M KCl, pH 7.4 (21). Aliquots of supernatant (0.05 ml) were mixed with 0.15 M Tris-HCl buffer, pH 7.4 (1 ml), containing acetylated cytochrome c (1 mg/ml) and NADPH, 1 mM. In all experiments dicoumarol was used at a concentration of 30 \( \mu \)M and SOD at 0.1 mg/ml. SOD-sensitive reduction of ferricytochrome derivatives cannot be used as an absolute measure of \( \cdot \text{O}_2^- \) production due to other competing reactions of \( \cdot \text{O}_2^- \), such as enzymatic and nonenzymatic dismutation. Protein was determined according to the method of Lowry et al. (17).

RESULTS

Alkylation reactions of quinones generally proceed at the ortho position to the quinone moiety. Thus both the 2,3-dialkylated derivatives (DMNQ and DENQ) would not be expected to directly alkylate biological nucleophiles. This was verified experimentally using GSH as a model nucleophile. If conjugate formation between quinone and GSH did not occur then full recovery of thiol equivalents in terms of GSH remaining and GSSG formed should be possible. That this was the case during reaction of DMNQ and DENQ with GSH is shown in Table I.

The toxicity of menadione and its two alkylated analogs, DMNQ and DENQ, in isolated rat hepatocytes is shown in Fig. 1 (A–C). The results show that both of the substituted analogs were markedly less toxic than menadione. The lack of toxicity of DMNQ and DENQ could reflect their inability to alkylate cellular nucleophiles but may also be a consequence of diminished one-electron reduction by mitochondrial and microsomal reductases relative to menadione. This would in turn lead to a decreased generation of semiquinone radical, and active oxygen species such as \( \cdot \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \). Alternatively, the diminished toxicity of DMNQ and DENQ may be due to their higher affinity for the enzyme DT-diaphorase [NADPH (quinone acceptor) oxidoreductase], a two-electron reductase which catalyzes the reduction of quinones to hydroquinones without the generation of semiquinone radical intermediates (18). Thus, it has been proposed that this enzyme plays a protective role in quinone-induced toxicity (1, 19). A dialyzed whole liver homogenate, devoid of soluble cellular thiols (which may interact with quinones directly to generate \( \cdot \text{O}_2^- \) (4, 20)), was used to investigate these possibilities. A decreased capacity of DMNQ and DENQ relative to menadione to generate species which caused acetylated cytochrome c reduction was evident using the dialyzed whole liver homogenate preparation (Fig. 2A). Reduction of the cytochrome can reflect generation of either hydroquinone\(^3\) or \( \text{O}_2^- \), as both

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>GSH remaining (( \mu )M)</th>
<th>GSSG formed (( \mu )M GSH eq)</th>
<th>Total (GSH eq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without quinone</td>
<td>170</td>
<td>31</td>
<td>201</td>
</tr>
<tr>
<td>DMNQ</td>
<td>143</td>
<td>66</td>
<td>209</td>
</tr>
<tr>
<td>DENQ</td>
<td>138</td>
<td>66</td>
<td>204</td>
</tr>
</tbody>
</table>

\(^*\)In Krebs-Henseleit buffer, pH = 7.4.
in Fig. 2. NADPH-dependent reduction of acetylated cytochrome c induced by 1,4-naphthoquinones in dialyzed whole liver homogenate. (Menadione (●); 2,3-dimethyl-1,4-naphthoquinone (○); and 2,3-diethyl-1,4-naphthoquinone (●)). (A) Concentration dependence of NADPH-(1 mM) dependent acetylated cytochrome c reduction in dialyzed whole liver homogenate. (B) Effects of SOD (0.2 mg/ml, open bar), and dicoumarol (0.03 mM, solid bar) on NADPH-dependent reduction of acetylated cytochrome c in dialyzed whole liver homogenate. (a) Menadione, 0.2 mM; (b) 2,3-dimethyl-1,4-naphthoquinone, 0.2 mM; (c) 2,3-diethyl-1,4-naphthoquinone, 0.2 mM. Means ± standard deviation of three experiments.

are capable of transferring electrons to acetylated cytochrome c. (1). With menadione, however, 50% of the reduction was SOD sensitive, suggesting the involvement of O₂, whereas SOD had no significant effect on DMNQ- and DENQ-induced acetylated cytochrome c reduction (Fig. 2B). Conversely, dicoumarol, an inhibitor of DT-diaphorase (19), almost totally inhibited the NADPH-dependent reduction of the cytochrome by DMNQ and DENQ, whereas menadione-induced reduction was still 35% of the control value (Fig. 2B). These experiments demonstrate that the alkyl-substituted analogs of menadione have a higher ratio of two- as opposed to one-electron reduction relative to menadione itself. In agreement with previous studies (1, 21) and the protective role of DT-diaphorase, pretreatment of hepatocytes with dicoumarol (0.03 mM) markedly exacerbated the toxicity of all three quinones to hepatocytes (data not shown).

In an attempt to clarify the role of oxidative processes in naphthoquinone-induced toxicity we have used cells pretreated with BCNU, an inhibitor of glutathione reductase. This treatment exacerbates intracellular oxidative challenge, as any GSSG generated in the cell cannot be reduced back to GSH. Both DMNQ and DENQ caused some loss in hepatocyte GSH content (Figs. 3B–C) relative to controls (Fig. 3A). There was, however, a potentiation of GSH removal of GSSG generation in BCNU-pretreated cells relative to control cells in the case of all three naphthoquinones (Figs. 3A–D). Furthermore, the three naphthoquinones were significantly more toxic to BCNU-pretreated cells than to control cells (Figs. 4A–D), demonstrating that enhanced oxidative challenge can result in enhanced cellular toxicity.

DISCUSSION

In this study we have investigated the metabolism and toxicity of alkyl-substituted analogs of menadione which cannot alkylate cellular nucleophiles directly in order to determine the role of oxidative processes in cytotoxicity. The alkyl-substituted analogs of menadione, DMNQ and DENQ, were markedly less toxic to isolated hepatocytes than the parent compound. High concentrations of DMNQ and DENQ were required to induce significant cytotoxicity to hepatocytes. These data do not allow us to conclude, however, that oxidative processes are unimportant in menadione-induced cytotoxicity and that alkylation reactions represent the only determinants of the toxicity observed. An alternative explanation for the decreased toxicity of DMNQ and DENQ relative to menadione may be their low ability to generate oxidizing species within the cell—either because of low affinity for one-electron reductases or because of higher affinity for alternative pathways of metabolism such as DT-diaphorase-catalyzed two-electron reduction. Using dialyzed liver homogenate we found that SOD could substantially inhibit menadione-induced acetylated cytochrome c reduction, whereas it had minimal inhibitory effect on DMNQ- and DENQ-mediated reduction (Fig. 2). This suggests that O₂ generated by redox cycling reactions is responsible for a substantial proportion of the observed reduction of
Whether a particular quinone is metabolized by one- or two-electron reductases depends on a number of factors such as steric considerations, lipophilicity, and one-electron reduction potential. The dependence of the rate of \( \text{O}_2 \) production by isolated rat hepatocytes on the one-electron reduction potential of quinones has been studied previously by Powis et al. (22). These workers showed that in isolated hepatocytes maximal quinone-induced \( \text{O}_2 \) formation occurs at a quinone one-electron reduction potential of \(-70\) mV (22). From their data one can also predict that a more negative one-electron reduction potential

acetylated cytochrome c only with menadione. Furthermore, data obtained using dicoumarol, an inhibitor of DT-diaphorase, showed that two-electron reduction was involved in the metabolism of all three naphthoquinones, but that this metabolic pathway was more active with DMNQ and DENQ. The alkylated analogs, therefore, undergo a higher ratio of two- as opposed to one-electron reduction relative to menadione and thus this represents a possible explanation of the decreased toxicity of DMNQ and DENQ relative to menadione.

Fig. 3. GSH depletion (a) and GSSG generation (b) during metabolism of 2,3-dimethyl-1,4-naphthoquinone 0.2 mM (B), 2,3-diethyl-1,4-naphthoquinone 0.2 mM (C), and menadione 0.05 mM (D), in control (○) and BCNU-pretreated cells (●). The thiol status of control (○) and BCNU-pretreated (●) cells in the absence of quinone but in the presence of the solvent vehicle (DMSO) is shown in (A). One experiment typical of three.

Fig. 4. Toxicity of 2,3-dimethyl-1,4-naphthoquinone 0.2 mM (B), 2,3-diethyl-1,4-naphthoquinone 0.2 mM (C), and menadione 0.05 mM (D), in control (○) and BCNU-pretreated cells (■). The toxicity of the solvent vehicle to control (○) and BCNU-pretreated (■) cells is shown in (A). One experiment typical of five.
than that of menadione (−200 mV) would lead to a marked decrease in the rate of superoxide formation. As alkyl substitution of the naphthoquinone nucleus would be expected to cause donation of electrons into the ring system, the one-electron reduction potentials of DMNQ and DENQ would be predicted to be more negative than menadione itself. Thus, on the basis of one-electron reduction potential alone, the alkyl-substituted derivatives of menadione would be expected to induce the generation of fewer oxygen-derived radicals.

The observation that DMNQ and DENQ were cytotoxic, albeit at high concentrations, suggests that oxidative processes alone can cause cytotoxicity. To investigate this we used cells preincubated with BCNU, which inhibits cellular glutathione reductase. In control cells, all three naphthoquinones induced some GSH loss and GSSG formation, but these changes were markedly exacerbated by BCNU pretreatment (Fig. 3), showing that the alterations in GSH homeostasis observed occur, at least in part, by means of oxidative mechanisms. Although DMNQ and DENQ did not induce significant formation of \( \cdot O_2^- \) in dialyzed liver homogenate (Fig. 2), naphthoquinones can still induce an oxidative challenge including \( \cdot O_2^- \) and \( H_2O_2 \) generation, via chemical reactions (23), and this may represent the route whereby DMNQ and DENQ induce oxidative stress.

As well as a potentiation of naphthoquinone-induced GSH loss and GSSG formation in BCNU-pretreated cells relative to control cells, marked potentiation of toxicity in the nitrosourea-treated cells was also observed. It seems probable that the potentiation of oxidative challenge observed in BCNU-pretreated cells contributes to the increased toxicity of the naphthoquinones, although our data is not conclusive proof of this. It is clear from the data shown in Figs. 3D and 4D that GSH loss alone does not necessarily result in cytotoxicity, thus confirming previous work (5).

When both alkylation and oxidation can play a role in quinone-induced cytotoxicity, for example in the case of 1,4-naphthoquinone (21) and menadione (5), the dissection of the relative importance of each process for the eventual cytotoxic effect observed is not a simple exercise. Indeed, as the alkylation of thiols by menadione (4, 20) and 1,4-naphthoquinone (G. Cohen, personal communication) produces active oxygen species, then the two processes are not mutually exclusive. In addition naphthoquinones may induce cytotoxicity by other undefined processes. Thus, it seems obvious that in the case of menadione and 1,4-naphthoquinone more than one mechanism contributes to cytotoxicity.

In this study we have shown that derivatives of menadione which cannot directly alkylate nucleophiles are still cytotoxic, albeit at high concentrations, in isolated hepatocytes. We have also investigated the toxicity of naphthoquinones in BCNU-pretreated cells. Increased quinone-induced changes in soluble thiol oxidation were observed in BCNU-pretreated cells and these changes were associated with exacerbated cytotoxicity. These data suggest that oxidative processes alone can function as a determinant of the cytotoxicity of naphthoquinones.

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