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INDUCTION OF DNA DAMAGE BY MENADIONE (2-METHYL-1,4-NAPHTHOQUINONE) IN PRIMARY CULTURES OF RAT HEPATOCYTES

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Abstract—The cytotoxicity of menadione (2-methyl-1,4-naphthoquinone) had been investigated using primary cultures of rat hepatocytes. Menadione was found to induce DNA strand breaks which were actively repaired by the cells. Dicoumarol, an inhibitor of DT diaphorase, did not potentiate menadione-induced DNA strand breaks. Neither had metyrapone, an inhibitor of cytochrome P-450 dependent monooxygenases, any effect on the extent of DNA damage. Covalent binding of menadione metabolite(s) to DNA was detected in the cultured hepatocytes and, in addition, hepatic microsomes were also found to metabolize menadione to DNA-binding products. The extent of binding of menadione to DNA *in vitro*, was markedly decreased by inclusion of the hepatic cytosol fraction, or reduced glutathione, in the incubations. In the presence of dicoumarol, menadione was also found to induce cell membrane damage. It also caused a rapid loss in cellular glutathione which was augmented by the presence of dicoumarol. The results suggest that both the cell membrane damage and DNA damage induced by menadione are mediated by one-electron reduction of the quinone to free radical intermediate(s). DT diaphorase appears to protect the cell from membrane damage, whereas reduced glutathione may have an important role in the prevention of DNA damage.

Quinones are widely distributed in nature and form an important group of substrates for flavoenzymes. They can undergo either one-electron reduction to yield the semiquinone radical, or two-electron reduction directly to the more stable hydroquinone [1, 2]; the cytotoxic and antitumour properties of quinoid drugs are thought to be mediated through the one-electron reduction to semiquinone free radicals [3, 4]. Most semiquinones are readily re-oxidized in aerobic conditions and can enter redox cycles with molecular oxygen, forming various reactive oxygen species such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide, hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^{1\Delta g}O_2$) [2]. Flavoprotein-catalyzed redox cycling of quinones can thus rapidly lead to conditions of oxidative stress in various cell types. For instance, exposure of isolated rat hepatocytes to menadione (2-methyl-1,4-naphthoquinone) results in production of superoxide, oxidation of glutathione (GSH) and loss of cell viability [5].

Reactive oxygen species produce DNA damage in bacteria, viruses and cells of mammalian origin [6–10]. In addition, semiquinones can also interact with DNA, for example by hydrogen abstraction, which can readily lead to a strand scission and cross-linking [9]. However, relatively little is known about the induction of DNA damage in mammalian cells by quinones and/or their metabolites. Therefore, we have investigated the ability of menadione to cause DNA damage in non-proliferating primary cultures of rat hepatocytes.

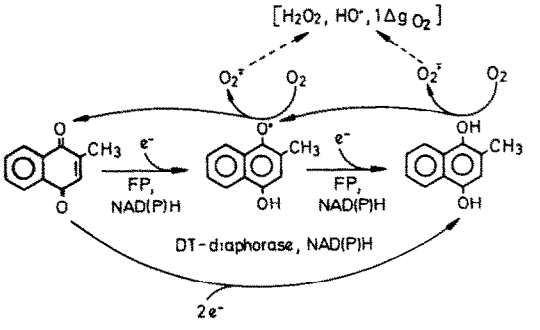

Fig. 1. Schematic representation of one- and two-electron reduction of menadione in hepatocytes. Fp, flavoprotein.

The toxicity of menadione in this system will be influenced by the relative contribution of one-electron or two-electron reduction to the overall metabolism of the quinone (see Fig. 1). The flavoprotein NAD(P)H: (quinone acceptor) oxidoreductase (EC 1.6.99.2), also known as DT-diaphorase, catalyzes the two-electron reduction of menadione directly to the hydroquinone, whereas NADPH-cytochrome P-450 reductase (EC 1.6.2.4), NADH-cytochrome b_5 reductase (EC 1.6.2.2) and NADH-ubiquinone oxidoreductase (EC 1.6.5.3) can catalyze the one-electron reduction of the quinone to the reactive semiquinone free radical [1, 2, 11]. DT-Diaphorase may therefore be able to protect the cells from the toxic effects of menadione by competing with the one-electron reduction pathways.

The relative contribution of DT-diaphorase and NADPH-cytochrome P-450 reductase to menadione-induced cytotoxicity in cultured hepatocytes can be

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affected by administration of either phenobarbital (PB) or 3-methylcholanthrene (MC) to the animals prior to cell isolation. MC pretreatment increases the activity of hepatic cytosolic DT-diaphorase but has little or no effect on NADPH-cytochrome P-450 reductase [12], whereas PB administration induces the hepatic NADPH-cytochrome P-450 reductase without affecting the activity of DT-diaphorase [12, 13]. In addition, the activity of DT-diaphorase is sensitive to inhibition by dicoumarol, whereas that of NADPH-cytochrome P-450 reductase is not [14]. The contribution of cytochrome P-450 dependent mixed function oxidase (MFO) activities to the formation of cytotoxic menadione metabolites was also investigated by including metyrapone in some incubations. This agent is a potent inhibitor of the MFO enzymes, but has little or no effect on NADPH-cytochrome P-450 reductase [15].

MATERIALS AND METHODS

Treatment of rats and preparation of cultured hepatocytes

Hepatocytes were isolated from male Sprague–Dawley rats (200–250 g) by collagenase perfusion as previously described [16]. Where indicated, rats were pretreated with either PB (1 mg/ml in the drinking water for 5 days before use) or MC (50 mg/kg i.p. given 3 days prior to cell isolation). The viability of the cell suspension was determined by Trypan Blue exclusion (typically 90–100%).

Primary cultures of hepatocytes were isolated using the conditions and medium previously described [17]. Within 30 min of preparation the cells were washed with sterile Krebs–Hepes buffer, pH 7.4, and resuspended in prewarmed medium (10^6 cells/5 ml medium). 5 ml aliquots of this cell suspension were dispensed into collagen precoated 60 mm tissue culture dishes [17]. The cells were incubated for 2 hr in a humidified atmosphere (5% CO_2/air) at 37° and during this period the cells attached to the petri dishes and formed a virtually continuous monolayer. All the experiments with menadione were carried out at this time in culture.

Briefly, the medium was based on Earle's balanced sodium salt supplemented with BME vitamins, RPMI 1640 amino acids (modified as previously described [17]), hydrocortisone-21-succinate 100 μM, 5-aminolaevulinic acid 100 μM, 5% (v/v) foetal calf serum and 1 IU/l zinc protamine insulin.

Incubation conditions

After 2 hr in culture the medium was removed by aspiration and replaced with 2 ml fresh medium. The cells were incubated in 60 mm petri dishes at 37° in an atmosphere of air/5% CO_2. Cultures (approx. 10^6 cells/petri dish) were exposed for 30 min to concentrations of menadione from 1 to 50 μM in the absence or presence of 30 μM dicoumarol. Both menadione and dicoumarol were added to the medium in dimethylsulfoxide (DMSO) (final concentration 1% v/v). The DMSO concentration was constant at all menadione concentrations and DMSO was present in the controls. At the end of the incubation time samples were immediately transferred to ice and the medium rapidly removed.

Analytical methods

Lactate dehydrogenase (LDH) activity. LDH activity was measured in the medium (50–100 μl) at the end of the incubation period [16, 18]. Measurement of viability by release of LDH into the medium allows determination of both cell membrane toxicity and DNA damage or cellular GSH content in cells from the same culture dish.

Determination of GSH, glutathione disulfide (GSSG) and protein content. For measurement of intracellular GSH and GSSG the cultured hepatocytes were washed once with Krebs–Hepes buffer, pH 7.4, and then resuspended in 2 ml of the same buffer by scraping with a 'rubber policeman'. A 1.0 ml aliquot was taken for determination of GSH and GSSG by the fluorimetric method of Hissin and Hilf [19]. The protein concentration was determined from the remaining cells by the method of Lowry *et al.* [20]. GSH and GSSG in the incubation medium were determined by the high pressure liquid chromatography (HPLC) method described by Reed and Beatty [21].

Measurement of DNA strand breaks. DNA strand breaks were determined according to the method of Ahnström and Erixon [22] modified as follows: after incubation the cells were washed once with ice-cold Krebs–Hepes buffer, pH 7.4, and then incubated in the dark in 3 ml ice-cold 0.03 M NaOH–0.97 M NaCl for 30 min. This was followed by adjustment to pH 6.8 by the addition of approx. 1 ml of 0.067 M HCl–0.02 M NaH_2PO_4. After sonication with a Branson sonifier (Mark II, 15 secs) and addition of sodium dodecyl sulphate (2.5 mg/ml, final concentration), the cells were scraped off the dishes, incubated with pronase (1 mg/ml), and the DNA chromatographed on 0.5 ml columns of hydroxylapatite. Single-stranded DNA was eluted with 4 ml 0.1 M potassium phosphate buffer, pH 6.8, and double-stranded DNA with 4 ml 0.25 M potassium phosphate buffer, pH 6.8. Both single- and double-stranded DNA were quantified using the fluorochrome Hoechst 33258 as described previously for human lymphocytes [23]. Alterations in the number of DNA strand breaks are expressed as the percentage of total fluorescence associated with the single-stranded fraction. The appropriate background fraction of single-stranded DNA has been subtracted in the figures. The background of approx. 25% single-stranded DNA is responsible and related to the denaturation conditions used.

Investigation of the repair of DNA strand breaks

Cells cultured from PB-treated rats were exposed to 50 μM menadione for 30 min at 37°. Thereafter the medium was removed, replaced with fresh medium and the incubations continued at 37°. Culture samples were taken at the times indicated for determination of the single-stranded DNA fraction as before.

Detection of covalent binding of menadione to hepatocyte DNA

For the detection of menadione covalently bound to DNA hepatocytes from PB-treated rats were cultured on 150 × 25 mm culture dishes coated with 5 mg of collagen. 6×10^6 cells in 20 ml medium were

added to each plate, and after 2 hr the medium and dead cells were removed by aspiration.

The cell cultures were exposed, for 30 min, to 50 μM [5, 6, 7, 8-^{3}H]-menadione (sp. act. 1010 dpm/pmole) in the presence of 30 μM dicoumarol. At the end of the incubation the medium was removed and the cells washed 5 times with Krebs–Hepes buffer, pH 7.4. Using a 'rubber policeman' the cells from 5 plates were scraped into 3 ml 1% Na-sarcosylate in 10 mM EDTA and incubated with 100 μg of proteinase K for 1 hr at 37°. The hepatocyte DNA was then purified and isolated, and then covalent binding of [^{3}H] menadione calculated as described by Dock *et al.* [24].

Covalent binding of [^{3}H]*menadione to calf thymus DNA*

Modifications in the extent of covalent DNA binding induced by menadione were investigated *in vitro* using 1 mg calf thymus DNA in a buffer composed of 50 mM Tris–Cl, 25 mM KCl and 5 mM EDTA (TKE), pH 7.0. Incubations contained 1 mg hepatic microsomal protein isolated from PB-treated rats [25], in the presence or absence of 1 mM NADPH; 1 mM GSH; 30 μM dicoumarol and 2.5 mg of hepatic soluble fraction from control rats [25]. The final volume of the incubation mixture was adjusted to 1.0 ml with TKE buffer, and the tubes preincubated for 2–3 min at 37°, before 50 μM [5,6,7,8-^{3}H]menadione (sp. act. 600 dpm/pmole) was added. After 30 min incubation, 0.1 ml of 10% Na-sarcosylate in 100 mM EDTA and 20 μl of a proteinase K solution (1 mg/ml) were added and the samples further incubated for 1 hr at 37°. Thereafter samples were extracted twice with an equal volume of phenol/chloroform (1:1 v/v) at 5° to remove proteins. To the remaining water phase, 0.2 ml of 6 M NaCl were added and the DNA precipitated by addition of 2 ml cold (−40°) ethanol. The precipitated DNA was washed twice in 70% ethanol, dissolved in 1 ml of SSC (15 mM NaCl, 1.5 mM Na citrate, pH 7.5) and the precipitation procedure repeated. DNA was finally dissolved in 1 ml of SSC, quantified by its absorbance at 260 nm, and mixed with 5 ml Lumagel scintillation cocktail and counted in LKB-Wallac 1216 Rackbeta Liquid scintillation counter with a counting efficiency of 40%. Covalent binding is expressed as pmole [^{3}H]menadione bound/mg DNA.

Materials

Dicoumarol, menadione, NADH, NADPH, GSH, calf thymus DNA, Hoechst 33258, 5-aminolaevulinic acid, collagen type III, proteinase K, and hydrocortisone-21-succinate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Hydroxylapatite (Biogel HTP) was from Bio-rad. Laboratories, Richmond, VA, U.S.A. [5,6,7,8-^{3}H]menadione (60 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, England. Earle's balanced salt solution, BME vitamin solution, and foetal calf serum were obtained from Gibco Biocult, Chemoform AB, Stockholm, Sweden. The tissue culture dishes were from Falcon, Labora AB, Stockholm, Sweden.

Statistics

Statistical evaluation of the data was carried out using the Wilcoxon–Mann–Whitney non-parametric test for two independent samples.

RESULTS

Figure 2 shows the increase in the fraction of single-stranded DNA induced by menadione in cells cultured from control, MC- and PB-treated rats. The cells from PB-induced rats were highly sensitive to menadione and a dose-dependent increase in the fraction of single-stranded DNA which showed a maximum response at 50 μM was observed. (At 75 μM menadione the increase in the % single stranded DNA was 27.6 ± 1.9%.) Addition of 0.5 mM of the cytochrome P-450 dependent monooxygenase inhibitor, metyrapone, to cells from PB-treated rats had no effect on the proportion of DNA strand breaks induced by 50 μM menadione. Due to the high sensitivity of cells from PB-treated rats to the menadione-induced DNA damage, they were used in all further experiments.

The effect of adding 30 μM dicoumarol, a specific inhibitor of DT diaphorase, to the incubations with menadione is illustrated in Fig. 3 in terms of induced DNA strand breaks (3A) and LDH activity (3B). The presence of dicoumarol did not enhance the effect of menadione on the fraction of single-stranded DNA. Under the incubation conditions used here, menadione alone did not cause cell membrane damage; however, in the presence of dicoumarol there was significant release of LDH activity from the cells at concentrations of menadione greater than 5 μM.

Figure 4 shows that the DNA strand breaks induced by menadione, in the absence of dicoumarol, are actively repaired. By 6 hr after exposure to 50 μM menadione the fraction of single-stranded DNA had returned to control values. Less than 3% of the total DNA fluorescence was lost after 6 hr indicating no significant loss of cells.

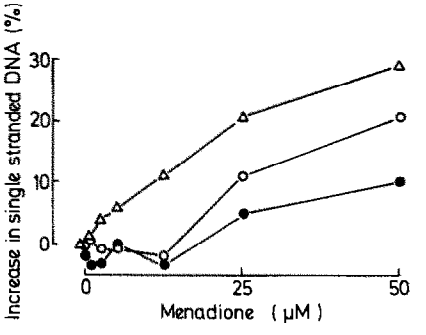

Fig. 2. DNA strand breaks induced by menadione in hepatocyte cultures from control (○), PB-induced (△) and MC-treated (●) rats. The appropriate background fraction of single-stranded DNA has been subtracted in each experiment. The mean background fractions of single stranded DNA were 24.9% in controls, 21.8% in PB and 26.2% in cells from MC treated rats. Results are the mean of 2 experiments, in which the data obtained at the same menadione concentrations differed no more than 7%.

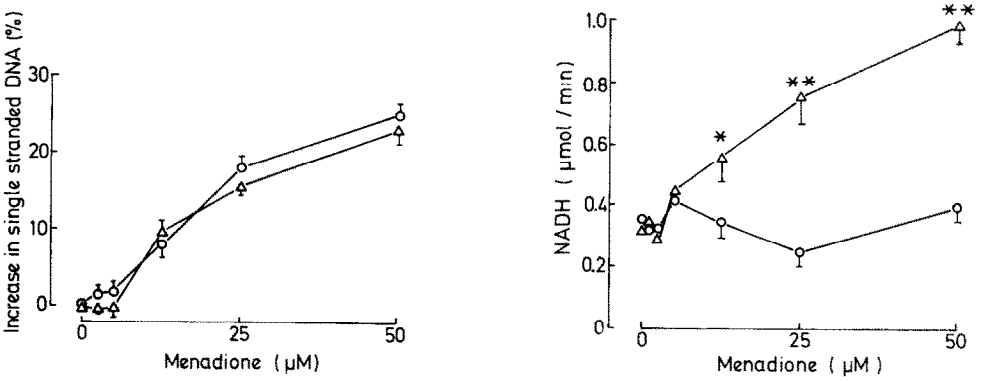

Fig. 3. (A) DNA strand breaks induced by menadione in the absence (○) and presence (△) of 30 μM dicoumarol in hepatocyte cultures from PB-treated rats. The appropriate background fraction of single-stranded DNA has been subtracted in each experiment. The mean background fraction of single stranded DNA was 22.4 ± 1.8% in the absence and 24.6 ± 1.1% in the presence of dicoumarol. Results are mean ± S.E. mean of 6 determinations. (B) LDH activity, expressed as μmoles NADH used/min/culture dish. Total LDH activity of fully lysed cells was about 1.5 μmoles NADH/min/dish. These data are derived from the same culture dishes as those in (A). **P = <0.005, *P = <0.05 by Wilcoxon–Mann–Whitney test. Significance values refer to differences between incubations in the presence and absence of dicoumarol.

The metabolism of menadione produces a concentration-dependent decrease in intracellular GSH level, as shown in Fig. 5. The presence of 50 μM menadione over 30 min decreased the GSH content from 35.3 ± 2.3 nmole/mg protein to 3.5 ± 0.8 nmole/mg protein. At concentrations lower than 2.5 μM menadione no apparent GSH depletion was observed. Intracellular GSSG content showed a slight increase only at 50 μM menadione (9.7 ± 2.6 nmole/mg protein compared to 6.0 ± 0.4 nmole/mg protein in control incubations). However, when the GSSG content of the incubation medium was analyzed by HPLC, 50 μM menadione was shown to induce a five-fold increase in GSSG concentration (see Fig. 6). The concentration of extracellular GSH in the medium varied between 1 and 5 μM and showed no relationship to menadione concentration. In the presence of dicoumarol, the intracellular GSH content fell more rapidly during menadione metabolism and, in parallel, there was a greater increase in extracellular GSSG concentrations (Figs. 5, 6).

The correspondence between depletion of cellular GSH and recovery of GSSG in the medium was good, particularly at concentrations of menadione exceeding 5 μM. At this concentration the GSH lost from the cells which could be accounted for by an increase in the total GSSG content (medium and

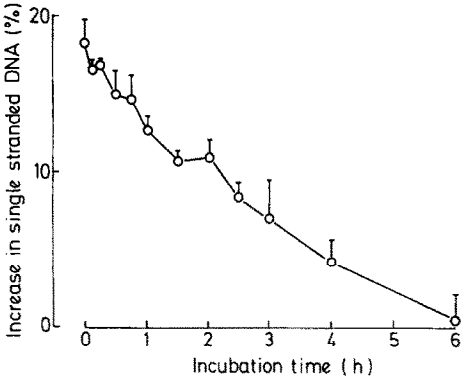

Fig. 4. Repair of DNA strand breaks induced by 50 μM menadione. PB-treated rats were exposed to menadione for 30 min then the medium was renewed (time zero) and the incubation continued for 6 hr. The appropriate background fraction of single-stranded DNA has been subtracted from each time point in each experiment. The mean background was 25.2 ± 2.1% single stranded DNA. Results are mean ± S.E.M. of 4 determinations.

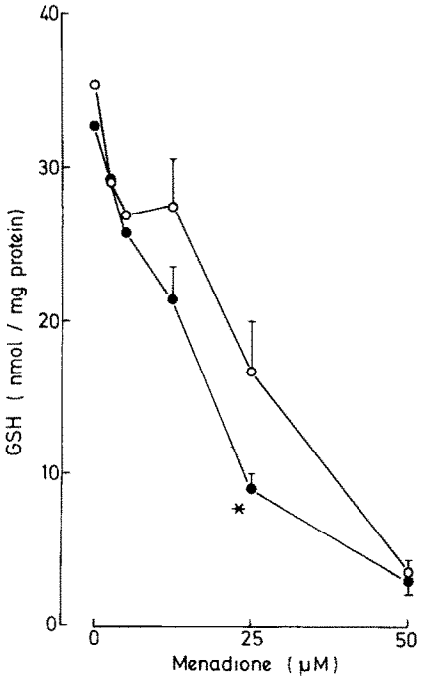

Fig. 5. GSH content of hepatocytes cultured from PB-treated rats exposed to menadione for 30 min in the absence (○) and presence (●) of 30 μM dicoumarol. Results are the mean ± S.E. mean of 4 or 6 determinations. *P = < 0.05 by Wilcoxon–Mann–Whitney test. Significance value refers to differences between incubations in the presence and absence of dicoumarol.

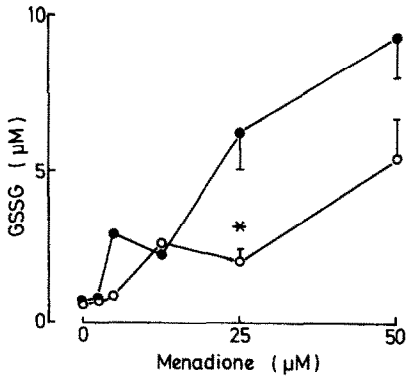

Fig. 6. Medium GSSG concentration after incubating liver cells from PB-treated rats with menadione for 30 min in the absence (○) and presence (●) of 30 μM dicoumarol. Results are the mean ± S.E. of 4 to 6 determinations. *P = < 0.05 by Wilcoxon–Mann–Whitney test. Significance value refers to differences between incubations in the presence and absence of dicoumarol.

intracellular GSSG) was 10% in absence and 75% in presence of dicoumarol. At 50 μM menadione the corresponding figures were 82% in absence and 100% in presence of dicoumarol. This indicates that the extent of oxidation of GSH increases with increasing concentration of menadione, and is greater in presence of dicoumarol. The discrepancy between GSH loss and GSSG recovery at low menadione concentrations may be explained by consumption of GSH by conjugation with menadione metabolites but at higher menadione concentrations oxidation of GSH appears to be more important than conjugation.

When binding of menadione to DNA was investigated in the cultured hepatocytes 5.2 pmole ^{3}H-menadione products were recovered bound per mg DNA in the presence of 30 μM dicoumarol. In order to characterize this binding further, *in vitro* experiments were performed using calf thymus DNA. This system is easily manipulated by the addition of subcellular rat liver fractions, such as microsomes and cytosol, and various cofactors and inhibitors. Table 1 shows the binding of [^{3}H]menadione when DNA was incubated with the soluble cytosolic fraction of control rat liver. There was no binding of [^{3}H] menadione over the background level detected in the presence of either NADH or NADPH to support

Table 1. DNA binding of [^{3}H]menadione catalyzed by hepatic cytosol

System	pmoles product bound/mg DNA
Cytosol + NADPH	−0.05 ± 0.50
Cytosol + NADH	−0.13 ± 0.67
Cytosol + NADPH + dicoumarol	−0.02, −0.49
Cytosol + NADH + dicoumarol	0.43, 0.77

Incubation conditions were as detailed in Materials and Methods. The background binding was 2.5 ± 0.47 pmoles/mg DNA (3 exp.) with cytosol alone and has been subtracted in such experiment. Where the numbers of experiments was 3 the mean ± S.E. is shown and where 2 experiments were carried out the individual values are shown.

Table 2. DNA binding of [^{3}H]menadione catalyzed by hepatic microsomes isolated from PB-treated rats

System	pmoles product bound/mg DNA
Microsomes + NADPH	6.62 ± 0.96
Microsomes + NADPH + dicoumarol	4.19 ± 1.10
Microsomes + NADPH + cytosol*	1.02 ± 0.87†
Microsomes + NADPH + cytosol + dicoumarol	2.85 ± 0.35
Microsomes + NADPH + GSH	0.76 ± 0.12†
Microsomes + NADH	5.31 ± 0.07
Microsomes + NADH + dicoumarol	4.46 ± 0.68
Microsomes + NADH + cytosol	0.68 ± 0.44†
Microsomes + NADH + cytosol + dicoumarol	3.03 ± 0.18‡
Microsomes + NADH + GSH	−0.41 ± 0.19†

* The cytosolic fraction contained 1 mM GSH. Incubation conditions were as detailed in Materials and Methods. The appropriate background (obtained by incubating microsomes + NADH or NADPH with [^{3}H]menadione followed by treatment with proteinase K and then addition of the DNA) has been subtracted in each experiment. Background binding was 3.43 ± 0.57 pmoles/mg with NADH and 5.29 ± 0.88 pmoles/mg with NADPH. Data are mean ± S.E. mean of 3 independent experiments.

† $P < 0.05$, by Wilcoxon–Mann–Whitney test. Significance values refer to differences between PB microsomes + NADPH or NADH in the presence and absence of either cytosol or GSH.

‡ $P < 0.05$. Significance value refers to the difference between PB microsomes + NADH + cytosol in the presence and absence of dicoumarol.

metabolism and, in addition, inhibition of DT-diaphorase did not result in any increased binding. Thus, cytosolic DT-diaphorase does not appear to be involved in the generation of reactive DNA-binding menadione intermediates.

Table 2 demonstrates that microsomes from PB-treated rats metabolize menadione to a DNA-binding species. Binding was dependent on the presence of either NADPH or NADH, both contributing about equally to the formation of the reactive species in this system. Dicoumarol did not significantly alter the amount of binding obtained with solely microsomes and NADPH or NADH. Addition of undialyzed cytosol from control rats markedly decreased the amount of binding in the presence of both NADH and NADPH, and this effect was only partially inhibited by dicoumarol. The inclusion of 1 mM GSH in incubations containing microsomes and either NADH or NADPH also resulted in an almost complete prevention of the DNA-binding.

DISCUSSION

DT-diaphorase catalyzes the reduction of various toxic quinones to hydroquinones, which are subsequently conjugated and detoxified [2]. A protective role for DT-diaphorase regarding the cytotoxicity of quinones was first suggested by Ernster *et al.* [2, 26, 27]. Supporting evidence has more recently been provided by Thor *et al.* [5], who demonstrated that menadione was toxic to rat hepatocytes in suspension as measured by uptake of Trypan Blue; the

toxicity of menadione was substantially higher in hepatocytes isolated from PB-treated rats compared with those from either control or MC-treated rats. This effect has been attributed to preferential induction by phenobarbital of NADPH-cytochrome P-450 reductase [12, 13] which results in a larger contribution of one-electron reduction to the overall metabolism of menadione and thus in an increased formation of reactive intermediates. Using hepatic subcellular fractions and purified enzymes it has been demonstrated that inhibition of DT-diaphorase with dicoumarol dramatically increases menadione-induced O_2 consumption and NADPH oxidation indicating the autooxidation of menadione semiquinone generated by NADPH-cytochrome P-450 reductase [2, 14]. Dicoumarol has also been shown to greatly potentiate the toxicity of menadione in isolated rat hepatocytes [5]. Under the experimental conditions used in the present study menadione was slightly toxic to cultured hepatocytes from PB-treated rats as revealed by an increased release of cellular LDH activity. However, the presence of dicoumarol greatly potentiated the menadione-induced cell membrane damage providing further evidence for a protective role of DT-diaphorase against quinone toxicity.

The experiments in the present study have shown that menadione also causes DNA-damage in cultured rat hepatocytes and that the effect is most pronounced in cells isolated from animals previously treated with PB (see Fig. 2). These observations are consistent with the participation and preferential induction of cytochrome P-450 reductase and stimulation of the one-electron reduction pathway. The addition of metyrapone had no effect on the proportion of single stranded DNA induced by menadione, thus indicating a negligible contribution from cytochrome P-450 dependent monooxygenase enzyme activities in the formation of DNA-damaging menadione intermediates. Further evidence for the involvement of one-electron reduction in menadione-induced DNA damage was obtained by incubating radioactive menadione and DNA under various conditions and measuring covalently bound radioactivity. Both NADH and NADPH support the microsomal activation of menadione to DNA-binding intermediates equally well. This observation was unexpected since the microsomal metabolism of menadione has previously been shown to be more efficiently supported by NADPH [5, 28, 12]. Although the identity of the particular binding species has not been established in this study, a semiquinone intermediate is a likely candidate. In fact, it has been suggested that semiquinones can bind to DNA [9, 29]. Cytosolic activities such as DT-diaphorase, were inactive with regard to the generation of DNA-binding menadione intermediates. This is not surprising as DT-diaphorase has been suggested to have a protective role during menadione induced toxicity [2, 5, 26, 27]. These results reflect the microsomal location of the enzymes activating menadione by one-electron reduction such as cytochrome P-450 reductase and cytochrome b_5 reductase. Cytosol containing 1 mM of GSH was found to almost abolish DNA-binding of menadione intermediates formed by microsomes, and this effect was only partially inhibited by dicoumarol. Furthermore, GSH itself almost completely prevents DNA-binding of the menadione intermediates. If GSH availability is a major determinant in DNA damage induced by menadione in the cultured hepatocytes, then it is unlikely that the presence of dicoumarol would effect the severity of such damage because, although dicoumarol potentiates the loss of cellular GSH, menadione itself also causes a rapid depletion of GSH (see Fig. 5). This is one possible explanation for the lack of effect of dicoumarol on the menadione-induced DNA damage in cultured hepatocytes.

An alternative explanation for the lack of effect of dicoumarol may be the existence of a spatial heterogeneity in the generation of DNA damaging menadione intermediates. It is possible that the inhibitory effect of dicoumarol on DT-diaphorase is not randomly distributed in the cell, rather the effect may be a function of the distance between the plasma membrane and the nucleus. This may offer an explanation for the potentiation of membrane damage in presence of dicoumarol as well as the lack of inhibition on DNA damage.

It would appear that both cell membrane damage and DNA damage are caused by products generated during the one-electron reduction of menadione. Although the identity of these reactive species is not known, this reaction generates both semiquinone free radicals and highly reactive species of oxygen. Reactive species of oxygen, including $O_2^{\cdot-}$, $OH^{\cdot}$ and H_2O_2 have been shown to be acutely toxic and DNA-damaging in microorganisms and mammalian cells [6–10, 30–32]. Moreover, within the cell, active forms of oxygen can also cause enzyme inhibition [33], lipid peroxidation [34] and oxidation of thiol groups in proteins [35].

It seems reasonable, therefore, to propose that $O_2^{\cdot-}$ and H_2O_2 resulting from one-electron reduction of menadione (see Fig. 1) are responsible for depletion of GSH [5, 26]. Up to a concentration of 2.5–5 μM menadione the hepatocytes were relatively resistant to menadione-induced GSH loss, and this could reflect saturation of the two-electron reduction pathway. Lind *et al.* [2] have demonstrated, using subcellular fractions of liver, that menadione is preferentially metabolized via DT-diaphorase to menadiol, and this is supported by the finding that in hepatic subcellular fractions the affinity of cytosolic DT diaphorase for menadione is almost ten-fold higher than that of the microsomal NADPH-cytochrome P-450 reductase [5].

In conclusion, both cell membrane damage and DNA damage induced by menadione appear to be mediated by the one-electron reduction pathway in cultured hepatocytes; however, the ultimate reactive species may differ. DT diaphorase seems to protect against the cell membrane damage but not against DNA damage measured as the formation of single stranded breaks. In addition, GSH prevents the DNA-binding of reactive metabolites of menadione.

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