Biochemical Pharmacology, Vol. 43, No. 10, pp. 2135–2142, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

MENADIONE-MEDIATED MEMBRANE FLUIDITY ALTERATIONS AND OXIDATIVE DAMAGE IN RAT HEPATOCYTES

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(*Received* 12 *August* 1991; *accepted* 20 *February* 1992)

Abstract—Menadione toxicity in isolated rat hepatocytes was mitigated by the antioxidant 4b,5,9b,10-tetrahydroindeno[1,2-*b*]indole at low concentrations (<100 μM), but not at high concentrations (>200 μM) of menadione. When hepatocytes were incubated with menadione, there was a time-dependent and concentration-dependent inhibition of lipid peroxidation in intact cells, as well as an increase in the antioxidative potency of acetone extracts, suggesting that metabolites of menadione could inhibit oxidative stress, and that at high menadione concentrations a different mechanism was involved in cytotoxicity. A possible mechanism was suggested by the ability of acetone extracts from hepatocytes that had been incubated with menadione to increase osmotic fragility in red blood cells. This increase correlated with an increase in membrane fluidity in red blood cells, determined by flourescence polarization using the membrane probe 1,6-diphenyl-1,3,5-hexatriene. At 200 μM menadione, an increase in membrane fluidity was also observed in hepatocytes. The thiol dithiothreitol protected hepatocytes from 50 μM menadione toxicity, but not from ≥100 μM menadione. The results suggest that while oxidative stress and arylation may be the critical mechanisms of toxicity at low menadione concentrations, at higher concentrations another mechanism such as enhanced membrane fluidity is operative.

Benzoquinones and related chemicals such as menadione are cytotoxic in hepatocytes and other cellular systems. These compounds possess an electrophilic carbon center and hence may bind cellular soft nucleophiles such as reduced glutathione (GSH‖) or protein thiols [1–4]. Such binding may produce toxicity directly if a critical protein thiol, such as a membrane cation transporter, is affected. Menadione binding to GSH may also produce a rapid and extensive decrease in GSH levels in hepatocytes which might predispose the cell to oxidative stress [3]. An alternate mechanism of producing oxidative stress involves the one-electron reduction of menadione to the semiquinone radical, catalyzed primarily by the microsomal NADPH–cytochrome P450 oxidoreductase and other flavoprotein reductases [5, 6]. Oxidation back to the parent quinone reduces molecular oxygen to superoxide anion in a redox cycle capable of generating large quantities of superoxide [7]. However, these intoxicating reactions compete with the two-electron reduction of quinones by NADPH: (quinone acceptor) oxidoreductase (quinone reductase or DT-diaphorase), followed by sulfation or glucuronidation, and excretion [8]. A protective role for quinone reductase was indicated by the potentiation of menadione toxicity by dicoumarol, an inhibitor of this enzyme [6, 9].

Menadione produces various biochemical lesions in hepatocytes which have been attributed to covalent binding to cellular thiols and to oxidative stress. However, it was observed [10] that in menadione-treated hepatocytes no lipid peroxidation occurred, despite a depletion of GSH and strong cytotoxicity. Even if the reactive oxygen species formed by menadione redox cycling could not initiate lipid peroxidation, the depletion of GSH would allow for lipid peroxidation to occur in dead and dying hepatocytes, as previously observed [11]. These considerations caused us to re-evaluate the nature of oxidative stress produced by menadione.

METHODS

Chemicals. Menadione, *tert*-butyl hydroperoxide (TBH) and semi-purified asolectin (soybean phospholipids) were obtained from the Sigma Chemical Co. (St. Louis, MO). Asolectin was purified and stored as previously described [12]. Molecular Probes, Inc. (Eugene, OR) supplied 1,6-diphenyl-1,3,5-hexatriene (DPH). All other chemicals were the highest quality available, and solvents were of reagent grade quality or better.

Hepatocytes. Male Wistar rats (*ca.* 225 g) were allowed food and water *ad lib*. Hepatocytes were prepared following collagenase (grade II, Boehringer

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‖ Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DTT, dithiothreitol; GSH/GSSG, reduced/oxidized glutathione; menadione, 2-methyl-1,4-naphthoquinone; RBC, red blood cell; TBARS, thiobarbituric acid reacting substances; TBH, *tert*-butyl hydroperoxide; TCA, trichloroacetic acid; and THII, 4b,5,9b,10-tetrahydroindeno[1,2-*b*]indole.

Mannheim, F.R.G.) digestion as described [13]. Cells were suspended in Krebs buffer containing 0.3% (w/v) HEPES, pH 7.4, and incubated in rotating round bottom flasks at 37° under a carbogen atmosphere. Menadione was added as a solution in dimethyl sulfoxide (DMSO) with the concentration of DMSO never exceeding 0.5%. Cell viability was assessed by trypan blue exclusion. The initial viability of hepatocyte preparations was 85–90%. Protein was determined using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) according to the technical procedure supplied by the manufacturer.

Lipid peroxidation. At various times after the addition of menadione or TBH, hepatocytes (1 mL) were treated with 0.1 vol. of 50% trichloroacetic acid (TCA) and centrifuged for 1 min at 2000 *g*; the cell pellet was suspended in 5 mL of 0.9% NaCl and centrifuged again. The resulting cell pellet was suspended in 1 mL of 1 M NaCl and extracted with 2 mL of ethyl acetate by sonication for 15 sec at 50% power, using a Kontes ultrasonic cell disrupter, followed by centrifugation at 10,000 *g* for 5 min. One milliliter of the ethyl acetate extract was dried under nitrogen and dissolved in 250 μL acetone. Aliquots of acetone or the ethyl acetate extract in acetone were added to a reaction mixture consisting of 1 mg/mL of purified soybean phospholipid vesicles prepared as described previously [12], 100 μM ascorbic acid and 10 μM ferrous ammonium sulfate. The reaction proceeded for 20 min at 25°, and was stopped by the addition of 0.01 vol. of 0.2 M butylated hydroxytoluene (to prevent artifactual TBARS that may arise in the heating stage of the assay) and 0.1 vol. of 50% TCA. After adding 0.67 vol. of 1% 2-thiobarbituric acid in 0.28% NaOH, samples were heated at 100° for 10 min, cooled and centrifuged for 5 min at 2000 *g*. The absorbance at 600 nm (correction for non-specific absorbance) was subtracted from absorbance at 532 nm in order to estimate thiobarbituric acid reacting substances (TBARS). For Fig. 2, malonaldehyde bis-dimethylacetal was used as the standard. It was verified that ≤50 μM 4b,5,9b,10-tetrahydro-indeno[1,2-*b*]indole (THII) did not affect the standard curve for the lipid peroxidation assay.

Red blood cell (RBC) osmotic fragility assay. The assay has been described previously [14]. Briefly, blood samples were removed from anesthetized rats into a heparinized syringe. Blood was diluted 20-fold with 140 mM NaCl, 10 mM (sodium citrate and 5 mM glucose, pH 7.4, and kept on ice no longer than 2 hr. DMSO (0–20 μL) or the ethyl acetate extract (described above) dissolved in DMSO was added to 0.75-mL aliquots of diluted blood. After 1 min and gentle swirling, 0.65 mL of distilled water was added forcefully in order to achieve rapid and complete mixing. Absorbance at 656 nm was used to monitor turbidity which is inversely proportional to the degree of osmolysis. The initial osmolysis in the absence of test compound was normally about 45%. The absorbance value for 100% lysis was obtained after diluted RBCs were sonicated for 5 sec at 50% power, using a Kontes ultrasonic cell disrupter.

Membrane fluidity. The fluorescent hydrocarbon DPH was used as a probe for monitoring fluidity in the various membrane preparations used in this study. This probe reports membrane fluidity independent of the cytoskeletal microtubules [15]. RBC membranes were labeled by adding 1 μL of 1 mM DPH in tetrahydrofuran per mL of membrane suspension containing 1 mg/mL protein. Suspensions of unlabeled membranes containing tetrahydrofuran without DPH were used as reference samples. Fluorescence was determined using a Shimadzu RF510LC equipped with polarizing filters on the excitation and emission sides. Determinations were made with the filters in parallel and perpendicular orientations, with excitation = 357 nm and emission = 428 nm. When using blood, excitation = 370 nm and emission = 460 nm, due to interference by hemoglobin at the standard wavelengths. Fluorescence intensity measurements were performed at 25°, 5–10 sec after exposing the samples to the excitation light. This prevented reversible bleaching of DPH that may result from photoisomerization that occurs after prolonged exposure to light [16]. The perpendicular component of fluorescence intensity was corrected for the intrinsic light polarization of the fluorometer [17], and the fluoroscence anisotropy (*r* value) was calculated from the equation: $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ [18], where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities in the parallel and perpendicular orientations of the polarizing filters, respectively.

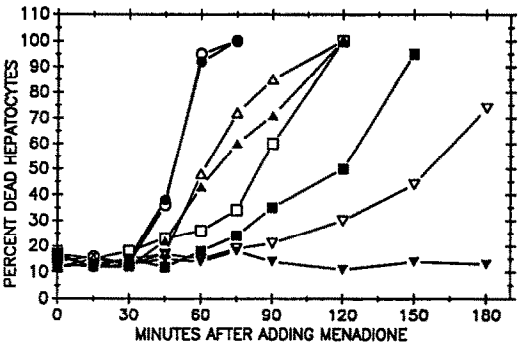

Fig. 1. Hepatocyte killing by menadione in the absence or presence of tetrahydroindenoindole (THII). Hepatocytes (10^6 cells/mL) were incubated in the presence of various concentrations of menadione [(▽, ▼) 50 μM; (□, ■) 100 μM; (△, ▲) 200 μM; (○, ●) 400 μM]. Five minutes prior to adding menadione, cells received either 5 μL DMSO/mL (open symbols), or a solution of THII in DMSO (closed symbols), such that the final THII concentration was 50 μM. In the absence of menadione, 50 μM THII or 5 μL DMSO/mL had no effect on cell viability. The results are representative of three separate experiments.

RESULTS

The cell viability curves in the presence of 0–400 μM menadione are shown in Fig. 1. Although the antioxidant THII protected against 50 μM menadione toxicity, the degree of protection

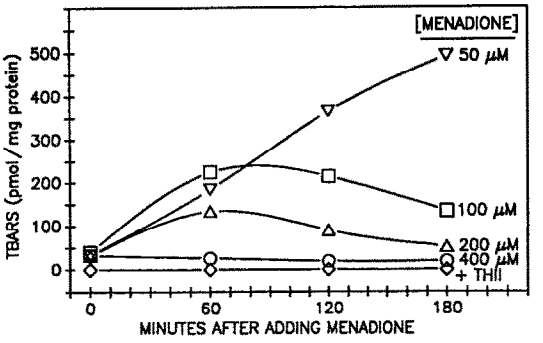

Fig. 2. Menadione-mediated thiobarbituric acid reacting substances (TBARS) in hepatocytes. Hepatocytes (10^6 cells/mL) were incubated with various concentrations of menadione and evaluated for the appearance of TBARS at 1-hr intervals. In the presence of 50 μM THII, menadione did not produce an increase in TBARS at any concentration (◇). In the absence of menadione, 5 μL DMSO/mL had no effect on TBARS. The results are representative of three separate experiments.

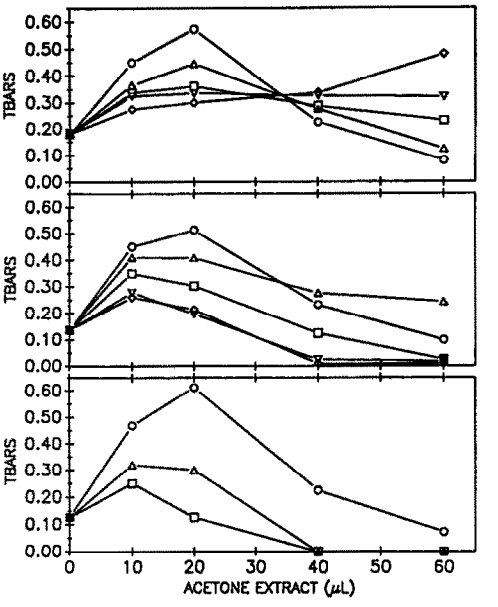

Fig. 3. Antioxidation potential of extracts of hepatocytes after incubation with menadione. Hepatocytes (10^6 cells/mL) were incubated with 100 μM (top panel), 200 μM (middle panel), or 400 μM (lower panel) menadione, which was added at zero min. Hepatocytes were treated with TCA immediately (○), or at 30 min (△), 60 min (□), 90 min (▽), or 120 min (◇) of incubation and then centrifuged, and the pellet was washed and extracted with ethyl acetate, as described in Methods. Aliquots of the dried extract dissolved in acetone were examined for their effect on the initial rate of lipid peroxidation in a cell-free system containing phospholipid, ascorbate and iron. TBARS are expressed as $A_{523\,nm}$ minus $A_{600\,nm}$, as described in Methods. The results are representative of two separate experiments.

diminished with increasing menadione concentration, such that no THII protection was evident at 400 μM menadione.

To determine whether lipid peroxidation, or other radical-mediated processes were related to cell killing by menadione, TBARS were evaluated (Fig. 2). Although the increase in TBARS preceded toxicity at lower menadione concentrations, increasing concentrations produced TBARS, with complete absence at 400 μM menadione. THII completely prevented the appearance of TBARS at any menadione concentration (Fig. 2, ◇).

In an attempt to determine whether the decrease in TBARS associated with increasing menadione concentrations in hepatocytes could be related to the appearance of low molecular weight antioxidants, hepatocytes were incubated with different concentrations of menadione for varying durations, followed by TCA treatment, centrifugation, and extraction of the cell pellet with acetone. This acetone extract was titrated into a cell-free system (described in Methods) to determine its ability to inhibit lipid peroxidation. Figure 3 indicates that when cells were sampled immediately after the addition of menadione (○), increasing amounts of acetone extract stimulated, and then inhibited, lipid peroxidation. As the duration of incubation with 100 μM menadione increased (top panel), the stimulation with smaller acetone aliquots, as well as the inhibition with larger aliquots, diminished. As the time of incubation and the concentration of menadione increased (middle and lower panels), the inhibitory phase became dominant.

If any of the results from Fig. 3 were due to oxidative stress imposed upon the cell by menadione, then perhaps those results could be modelled by repeating the experiments in the presence of a known oxidant, TBH. TBH produced a time-dependent killing of hepatocytes (Fig. 4, upper panel). At various durations of incubation, cells were extracted with ethyl acetate and dissolved in acetone (described above), and extracts were examined for their ability to inhibit lipid peroxidation in the cell-free assay system. The results (Fig. 4, lower panel) resembled those shown in Fig. 3 (top panel), especially with respect to the decrease in antioxidant efficacy of the extracts with increasing time.

It appeared that higher concentrations of menadione were generating antioxidants that partitioned into the membrane. We have observed previously that the hepatotoxicity of butylated hydroxytoluene (a hydrophobic antioxidant) was in part mediated by its ability to increase membrane fluidity, which was associated with an increase in membrane permeability [19]. Therefore, we utilized RBCs as a model system to evaluate the ethyl acetate extracts from hepatocytes incubated with menadione for their effect on membrane fluidity, and resistance of membranes to osmotic stress. Figure 5 (upper panel) shows that acetone extracts from cells incubated with increasing concentrations of menadione increased, in a concentration-dependent fashion, the sensitivity of RBCs to osmolysis imposed by the addition of water to an RBC suspension. This enhanced membrane fragility correlated with an increase in

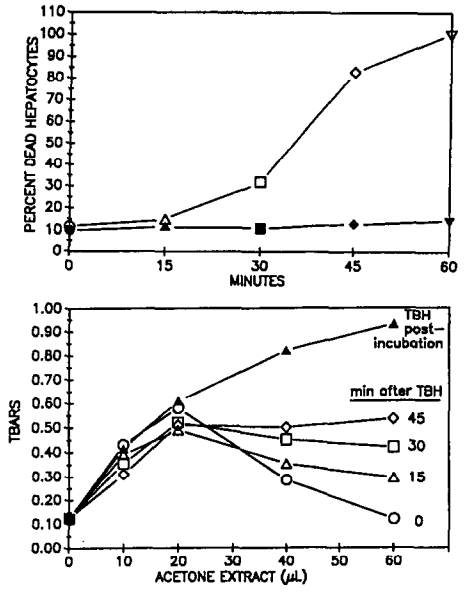

Fig. 4. Cell death and antioxidation potential of extracts of hepatocytes after incubation with *tert*-butyl hydroperoxide (TBH). Hepatocytes (10^6 cells/mL) were incubated in the presence (open symbols) or absence (closed symbols) of 0.75 mM TBH for the time periods indicated in the figure. This was followed by TCA treatment, centrifugation, washing and extraction of the pellet with ethyl acetate. Aliquots of the dried extract dissolved in acetone were examined for their effect on lipid peroxidation as described in Methods. For TBH-post incubation, hepatocytes were centrifuged and the cell pellet was sonicated for 1 min in the presence of 0.75 mM TBH. After 10 min of incubation with TBH, 0.1 vol. of 50% TCA was added and the mixture was centrifuged. The pellet was washed with 5 mL of 0.9% NaCl, centrifuged, and the pellet was extracted with ethyl acetate. TBARS are expressed as $A_{532\,nm}$ minus $Z_{600\,nm}$, as described in Methods. The results are representative of two separate experiments.

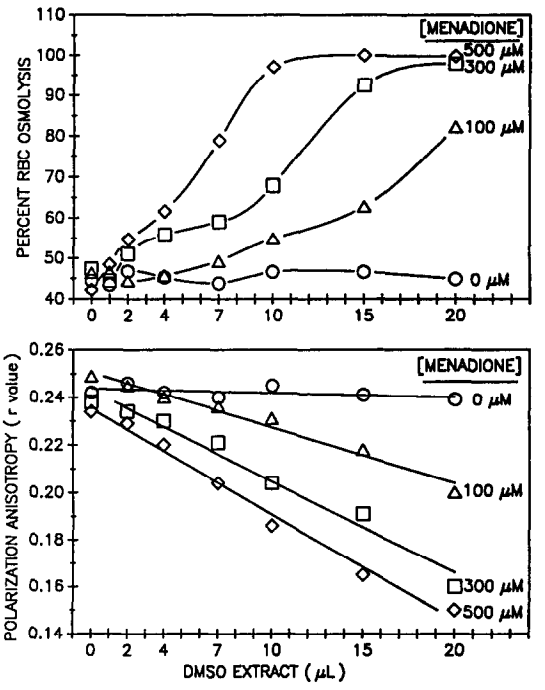

Fig. 5. Effects of extracts from hepatocytes incubated with menadione on RBC osmolysis and membrane fluidity. Hepatocytes (10^6 cells/mL) were incubated with 0 μM (○), 100 μM (△), 300 μM (□), or 500 μM (◇) menadione, which was added at zero min. After 60 min, DMSO solutions of ethyl acetate-extracted hepatocytes were prepared as described in Methods, and added to RBCs to determine effects upon RBC osmolysis and membrane fluidity. These procedures are described in Methods. The results are representative of two separate experiments.

membrane fluidity (decrease in r value) in RBCs incubated with acetone extracts from menadione-treated hepatocytes (Fig. 5, lower panel).

To determine whether similar effects occurred in hepatocyte membranes, the time courses for toxicity and membrane fluidity were followed in the absence and presence of 200 μM menadione (Fig. 6). In the absence of menadione (control), membrane fluidity decreased with time (increase in r value). However, menadione produced a time-dependent increase in hepatocyte membrane fluidity which preceded cell death.

Since low molecular weight thiols have been shown previously to protect against toxicity mediated by certain chemicals that exert their effects through redox cycling or arylation of critical protein cysteinyl residues [20–22], the ability of dithiothreitol (DTT) to prevent menadione toxicity was examined. Figure 7 indicates that DTT was an effective protectant against 50 μM menadione. However, at higher meoadione concentrations DTT was not protective.

To evaluate the possibility that menadione *per se* affected lipid peroxidation or RBC osmotic fragility, the compound was tested directly. Menadione

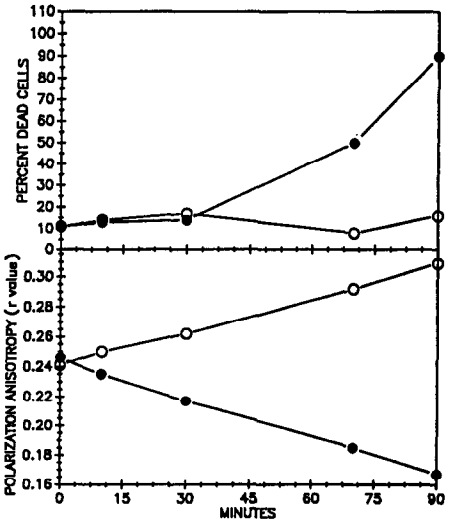

Fig. 6. Effects of menadione on cell killing and membrane fluidity in hepatocytes. Hepatocytes (10^6 cells/mL) were incubated for 30 min with 1 μL/mL of 1 mM DPH in tetrahydrofuran (THF), or with THF alone. At zero min, 200 μM menadione (●) or DMSO vehicle (○) was added. At the time points indicated, cells were evaluated for trypan blue uptake or polarization anisotropy. The results are representative of two separate experiments.

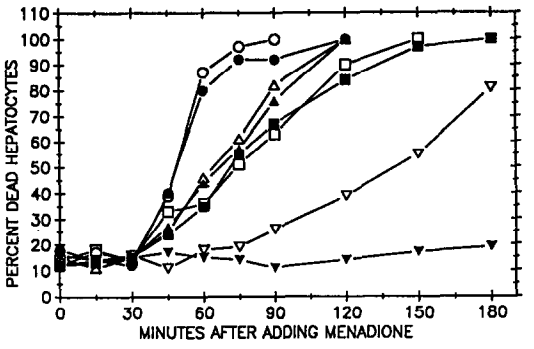

Fig. 7. Hepatocyte killing by menadione in the presence and absence of dithiothreitol (DTT). Hepatocytes (10^6 cells/mL) were incubated in the presence (closed symbols) or absence (open symbols) of 5 mM DTT. After 5 min, menadione was added at the indicated concentrations [(▽, ▼) 50 μM; (□, ■) 100 μM; (△, ▲) 200 μM; (○, ●) 400 μM]. In the absence of menadione, 5 μL DMSO/mL had no effect on cell viability. The results are representative of two separate experiments.

(≤500 μM) was without effect in the RBC osmotic fragility assay, and did not inhibit lipid peroxidation in a system [12] composed of vesicular phospholipid substrate and initiated by ferrous iron and ascorbate (data not shown).

DISCUSSION

Arylation and redox cycling are two different mechanisms that have been postulated to explain the cytotoxicity of quinone imines [21, 23, 24]. The electrophilic carbon *beta* to a carbonyl group renders these compounds sensitive to attack by soft nucleophiles such as the thiolate anion of GSH or protein thiols, producing a covalent adduct [1]. Alternately, the quinones and quinone imines may redox cycle to produce various reactive oxygen species [5, 25, 26].

Differentiating between arylation and oxidative stress as mechanisms involved in quinone and quinone imine cytotoxicity has been difficult. Using methyl substituted analogs of *N*-acetyl-*p*-benzoquinone imine or benzoquinone to prevent arylation, it was shown that both arylation and redox cycling may lead to cytotoxicity, but glutathione depletion is a prerequisite for cytoxicity with pure redox cycling (i.e. non-arylating) compounds [21, 22, 27].

Redox active naphthoquinones such as menadione (a quinonoid vitamin K analog) produce acute toxicity in isolated hepatocytes [2, 3, 6]. Menadione toxicity is preceded by a rapid depletion of GSH, 75% of which forms GSSG, an indicator of oxidative stress, while 25% forms menadione-SG conjugates and 10% forms mixed disulfides with protein cysteinyl residues [3]. A strict correlation was observed between the loss of protein thiols and cytotoxicity [2], similar to the results obtained with the free radical-mediated cytotoxic compound *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [28]. In the course of menadione toxicity, the ability of the cell to resist oxidative stress or arylation of protein thiols is compromised by an inhibition of GSSG reductase [29].

The data from the present study suggest that oxidative stress is the major cause of menadione toxicity at <100 μM, In Fig. 3, addition of small aliquots of hydrophobic ethyl acetate-extractable material from hepatocyte TCA precipitates stimulated lipid peroxidation, presumably due to the addition of peroxidizable lipid substrate. Addition of larger aliquots produced a response that varied with the concentration of menadione to which the hepatocytes were originally exposed. Exposure of hepatocytes to <100 μM menadione decreased the antioxidation potential of the ethyl acetate extracts, while exposure to higher menadione concentrations increased the antioxidation potential. Since vitamin E is the only highly reactive lipid soluble chain-breaking antioxidant operative at normoxic pO_2 [30] (β-carotene is active only at low pO_2 [31] and ubiquinone is a poor antioxidant [32]), it is reasonable to propose that vitamin E is responsible for the decrease in TBARS noted with larger aliquots of hepatocyte extract from zero time incubation with menadione. If vitamin E levels were diminished, this by itself could produce an increase in membrane fluidity and an associated increase in membrane permeability [33]. However, vitamin E depletion may not represent the entire mechanism by which membrane fluidity is increased in the presence of menadione, since cellular extracts from hepatocytes incubated with menadione produced a menadione concentration-dependent increase in membrane fluidity.

Regarding the nature of the hydrophobic antioxidant material produced by menadione in hepatocytes, a likely candidate would be the two-electron reduction product, the hydroquinone. As a class, hydroquinones are potent antioxidants [34, 35]. Menadione is reduced to the hydroquinone by NAD(P)H supported quinone reductase (DT-diaphorase), or by reductive addition of GSH to form the glutathionyl-hydroquinone conjugate [36]. Although the glutathionyl conjugate of menadione may redox cycle more rapidly than the parent compound in cell-free extracts, conjugation in the cell would favor partioning into the aqueous cytosolic phase and out of the membrane phase, thus allowing for quinone reductase to maintain the two-electron reduced state and inhibit redox cycling between the quinone and semiquinone states [36]. Therefore, the unconjugated menadione hydroquinone would be a likely antioxidant species that would continue to partition in favor of the hydrophobic membrane phase.

The titration curves for hepatocyte membrane extracts after incubation with TBH or 50 μM menadione were similar, indicative of the depletion of endogenous membrane antioxidants without significant antioxidant production from menadione. Finally the antioxidant THII [37] or the thiol DTT protected completely from 50 μM menadione toxicity, but poorly or not at all from 400 μM. At intermediary concentrations of menadione, DTT did not protect, while THII was decreasingly protective

at higher menadione concentrations. This may be explained in part by the hydrophobic nature of THII, with a log partition coefficient of 3.04, enabling THII to partition in favor of hepatocyte membranes, thus giving THII an advantage over DTT in preventing membrane-associated damage.

Furthermore, if arylation of critical toxicologic sites such as the Ca^{2+} translocase were responsible for toxicity, then the thiol DTT might have been expected to prevent $\geq 100\,\mu M$ menadione cytotoxicity. Such protection has been observed previously in the case of diamide and TBH [20] and quinone imines [21, 22], but not for menadione. However, it was reported [38] that GSH or DTT reverses the inhibition of the hepatocyte plasma membrane Ca^{2+}-ATPase activity produced by $200\,\mu M$ menadione. Since Fig. 7 demonstrates that DTT did not prevent hepatotoxicity from $200\,\mu M$ menadione, then it would appear that although arylation of the Ca^{2+}-ATPase occurs, it is not solely responsible for hepatotoxicity. In addition to the Ca^{2+}-ATPase, menadione has been shown to alkylate an essential free sulfhydryl group of the inositol triphosphate receptor of the rat hepatocyte plasma membrane [4], while not affecting the α_1-adrenergic receptor [39]. The toxicological implications for this effect are unclear.

We propose that a third mechanism for menadione toxicity is via an increase in plasma or mitochondrial membrane fluidity. Increased membrane fluidity in hepatocytes in the presence of $200\,\mu M$ menadione was evident at 30 min, before the onset of toxicity. Such fluidity increases would permeabilize these membranes, as has been shown previously for the cytotoxicant butylated hydroxytoluene [14, 40]. Conversely, fluidity decreases have been associated with reduced membrane lipid permeability [41]. The proposed increase in mitochondrial permeability would uncouple oxidative phosphorylation by increasing H^+ and/or Ca^{2+} permeability, resulting in a decrease in energy charge and redox state of the cell. Permeabilizing the plasma membrane to Ca^{2+} would produce toxicity similar to that observed following oxidative or arylating damage to the Ca^{2+} translocases, by virtue of mitochondrial uncoupling and a rise in intracellular Ca^{2+} in either case. A rapid increase in cellular Ca^{2+} levels by $200\,\mu M$ menadione has been observed previously [39]. This proposed mechanism would be consistent with the efficacy of THII to decrease 100–200 μM menadione toxicity, since THII has been shown to stabilize the RBC membrane and prevent osmolysis [14].

We conclude that menadione toxicity in hepatocytes proceeds by multiple mechanisms; oxidative stress and arylation dominate at low menadione concentrations, while increased membrane fluidity (associated with increased membrane permeability) is an additional mechanism that appears at higher menadione concentrations.

Acknowledgements—This work was supported by USPHS Grant ES-03373 and the Swedish Medical Research Council. The synthesis of THII was supported by AB Astra Hässle, Sweden.

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