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MECHANISMS OF PROTECTION FROM MENADIONE TOXICITY BY 5,10-DIHYDROINDENO[1,2,-*b*]INDOLE IN A SENSITIVE AND RESISTANT MOUSE HEPATOCYTE LINE

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Abstract—Established cell lines derived from newborn livers of c^{14CoS}/c^{14CoS} and c^{ch}/c^{ch} mice have been shown to be genetically resistant (*14CoS/14CoS* cells) or susceptible (*ch/ch* cells) to menadione toxicity. These differences are due in part to relatively higher levels of reduced glutathione (GSH) and NAD(P)H:menadione oxidoreductase (NMO1) activity in the *14CoS/14CoS* cells. The indolic membrane-stabilizing antioxidant 5,10-dihydroindeno[1,2-*b*]indole (DHII) was shown previously to protect against various hepatotoxicants *in vivo* and in primary rat hepatocytes. This report describes how the *14CoS/14CoS* and *ch/ch* cell lines provide a valuable experimental system to distinguish the mechanism of chemoprotection by DHII from menadione toxicity. The addition of 25 μM DHII produced a time-dependent decrease in menadione-mediated cell death in *14CoS/14CoS* cells, with little effect on *ch/ch* cell viability. The maximum protective effect occurred at 24 hr, although the concentration of DHII remained constant for 48 hr. The protective effect of DHII correlated with enhanced glutathione levels (234% increase at 24 hr), as well as induction of four enzymes involved in the detoxification and excretion of menadione: NAD(P)H:menadione oxidoreductase (NMO1, quinone reductase), glutathione reductase, glutathione transferase (GST1A1), and UDP glucuronosyltransferase (UGT1*06), with 24-hr maximum induction of 707, 201, 171 and 198%, respectively. Other biotransformation enzymes not directly involved in menadione metabolism (glutathione peroxidase, cytochromes P4501A1 and P4501A2, copper-, zinc-dependent superoxide dismutase, and NADPH cytochrome *c* oxidoreductase) were not induced by DHII. Menadione-stimulated superoxide production was inhibited 50% by DHII only in *14CoS/14CoS* cells, and the inhibition required 24-hr preincubation. Pretreatment with DHII also protected both cell types against the menadione-mediated depletion of GSH, and the increase in percent (oxidized glutathione GSSG), an indicator of oxidative stress. These results suggest that DHII does not protect against menadione toxicity by virtue of its antioxidant or membrane-stabilizing properties. Rather, it acts by inducing a protective enzyme profile that mitigates redox cycling and facilitates excretion of menadione.

Benzoquinones and related compounds are considered to be toxicologically important because of their biochemical reactivity and the potential for human exposure [1]. Such compounds may directly bind covalently with cellular soft electrophiles [2–4], generate superoxide and hydrogen peroxide via redox cycling and superoxide dismutase [5–7], or modulate membrane fluidity [8].

To examine mechanisms of toxicant-mediated damage, a model experimental system has been developed recently which consists of two SV40-transformed established hepatocyte cell lines that have marked differences in susceptibility to menadione. The *14CoS/14CoS* (resistant) and *ch/ch* (susceptible) cell lines were developed from newborn livers of the homozygous deletion mutant c^{14CoS}/c^{14CoS} mouse and the wild-type c^{ch}/c^{ch} mouse, respectively [9]. The several-fold elevated reduced glutathione (GSH§) levels and NAD(P)H:menadione (quinone) oxidoreductase (NMO1) activity in *14CoS/14CoS* cells, compared with *ch/ch* cells, appear to be related to the menadione resistance of these cells. The importance of GSH [3, 10, 11], and NMO1 [7, 12–14] in the protection against toxicity from quinones and quinone imines allows for the possibility of toxicity intervention by compounds that up-regulate these protective parameters.

Such a promising chemoprotectant is the synthetic indolic antioxidant, 5,10-dihydroindeno[1,2-*b*]indole (DHII) [15], which has been shown to protect *in vivo* and *in vitro* against a variety of hepatotoxicants, including carbon tetrachloride, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, methylmethane sulfonate, as well as menadione [16–20]. Chemoprotection by DHII and other hydrophobic protectants correlated with its membrane stabilizing properties [17]. DHII also enhanced GSH levels in Hepa-1c1c7 hepatocytes (unpublished observations), and was a potent hepatic inducer of certain biotransformation enzymes in rats and mice, including cytochrome P4501A1 (CYP1A1), NMO1 and glutathione transferase form 1A1 (GST1A1) [19, 20].

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§ Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; NMO1, NAD(P)H:menadione oxidoreductase [also called quinone reductase, DT-diaphorase, NAD(P)H:(quinone acceptor) oxidoreductase, and azo dye reductase]; GST1A1, glutathione transferase (Ya or class α); Cu,Zn-SOD, copper- and zinc-dependent superoxide dismutase; DHII, 5,10-dihydroindeno[1,2-*b*]-indole; and DMSO, dimethyl sulfoxide.

In earlier studies of the protective effects of DHII, it was not possible to clearly differentiate among the various possible direct and indirect mechanisms involved. In this paper we use the *14CoS/14CoS* and *ch/ch* cell models to show that immediate effects (radical quenching and modulation of membrane fluidity) are *not* responsible for the DHII-mediated protection from menadione damage. Rather, DHII is protective in *14CoS/14CoS* cells by virtue of inducing a protective enzyme profile that includes a marked elevation of NMO1 activities and GSH levels.

METHODS

Chemicals. All chemicals, reagents and enzymes used in this study were obtained from either the Sigma Chemical Co. (St. Louis, MO) or from the Aldrich Chemical Co. (Milwaukee, WI), except as noted below. DHII was synthesized as previously described [16].

Established cell lines. The generation of the established cell lines is described in detail elsewhere [9] and is described briefly here. Hepatocytes from *ch/ch* and *14CoS/14CoS* newborns were obtained by collagenase digestion of the mouse livers. Cells were infected at 33° (permissive temperature) with an SV40 *tsA255* mutant virus, conditionally transforming the cells that express the transformed phenotype at the permissive temperature. At the nonpermissive temperature of 40°, the cells revert to a normal nontransformed phenotype [21]. Cells are cultured at 33° for 5 weeks in α-Modified Eagle's Medium (MEM) supplemented with 4% fetal bovine serum and 0.1 μM dexamethasone. Identifiable clones of transformed cells were selected and recloned. Hepatocyte lines having high levels of albumin and transferrin were further characterized. A wild-type clone (20B, *ch/ch-1*) and a deletion homozygote clone (128, *14CoS/14CoS-1*) [22] were used in this study, and are referred to as *ch/ch* and *14CoS/14CoS*, respectively.

The two cell lines were grown at 34° in 75-cm^2 flasks containing 15 mL of Dulbecco's MEM supplemented with 5% fetal bovine serum, 0.1% gentamycin, and 26 mM $NaHCO_3$, under 95% air:5% CO_2. Cells in exponential growth (about 70% confluent) were used for these studies. Cell viability was assessed by rinsing the attached cells with wash buffer (5.4 mM KCl, 137 mM NaCl, 1 mM $MgSO_4$, 5.6 mM glucose and 25 mM HEPES-KOH, pH 7.4), followed by removal of attached cells from the culture flask with 0.05% trypsin containing 0.53 mM EDTA. The number of viable cells was then determined with a Coulter Counter (model ZM, Coulter Electronics).

Menadione and DHII were used as solutions in DMSO vehicle. For the viability study presented in Fig. 1, the concentration of MD was chosen independently for each cell type in order to produce approximately 50% viability after a 2-hr exposure and thereby compare the two cell types in the same sensitivity range. Thus, the MD concentrations used were 40 μM (*14CoS/14CoS* cells) or 20 μM (*ch/ch* cells). Cells were treated with 25 μM DHII or dimethyl sulfoxide (DMSO) vehicle, followed in some experiments by washing cells and treating further with fresh medium containing menadione in DMSO vehicle. The total DMSO concentration for both treatments never exceeded 0.5%, a concentration for which there were never observed effects on any of the parameters described in this study. Cellular content of DHII was determined using HPLC by the method of Haky and Young [23], using the following extraction procedure. Cells were washed three times with a buffer consisting of 25 mM HEPES, 5.36 mM KCl, 136.8 mM NaCl, 1 mM $MgSO_4$ and 5.6 mM glucose (pH 7.4). The cells were harvested by scraping the flasks with a rubber policeman and resuspended in 0.9% NaCl. Then, 0.1 M sodium lauryl sulfate, ethanol and hexane were added sequentially (1:3:3, by vol.), with vigorous vortexing of the cellular suspension following each addition. After centrifugation, the organic layer was removed and evaporated to dryness under a steady stream of nitrogen at ambient temperature. The residue was reconstituted in 1.0 mL ethanol, and 100 μL was injected into an HPLC fitted with a 4 μm NovaPak C-18 (8 mm × 10 cm) column. Using a mobile phase of methanol: methylene chloride:50 mM ammonium phosphate, pH 3.6 (82:13.5:4.5, by vol.) at a flow of 1.00 mL/min, DHII was detected by repeated scanning from 190 to 500 nm (3 nm resolution) of the eluate using a scanning photo-diode array detector. DHII was identified by the peak co-eluting with authentic standard, and verified by the comparison of ultraviolet/visible spectra.

Assays. Cells were rinsed with wash buffer and detached from flasks with 1 mM EDTA/1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), instead of trypsin/EDTA, in order to minimize membrane damage. The cells were centrifuged for 1 min at 500 *g* and resuspended in fresh wash medium. For superoxide formation, the assay medium contained 100 μM oxidized cytochrome *c*, 0.5 mM EDTA, 0.5 mM sodium azide, 25 mM potassium phosphate buffer (pH 7.4), and 0.5 mL cell suspension (final cell density of 3×10^5/mL). Menadione (5 μL, final concentration 80 μM) was added in the absence or presence of 20 units of superoxide dismutase (SOD)/mL. The rate of superoxide production was calculated as the SOD-inhibited rate of cytochrome *c* reduction, determined spectrophotometrically at 37°, using an extinction coefficient of 21.1 $cm^{-1}\,mM^{-1}$ for cytochrome *c* [24].

The activities of NMO1 (EC 1.6.99.2) [25], glutathione transferase (GST1A1, EC 2.5.1.18) [26], glutathione peroxidase (EC 1.11.1.9) [27], glutathione reductase (EC 1.6.4.2) [28], CYP1A1 as ethoxyresorufin *O*-deethylase (microsomal cytochrome P_1450; aryl hydrocarbon hydroxylase) [29], copper- and zinc-dependent SOD (Cu, Zn-SOD, EC 1.15.1.1) [30], and NADPH-cytochrome *c* oxidoreductase (cyt. *c* reductase, EC 1.6.2.4) [24] were assayed as described in the references cited. CYP1A2 (microsomal cytochrome P_3450, acetanilide 4-hydroxylase) was determined as described [31] except that we used normal phase thin-layer

chromatography with chloroform:methanol (80:20) as the mobile phase. Microsomal UGT1*06 (UDP glucuronosyltransferase) (EC 2.4,1.17) was assayed using *p*-nitrophenol as substrate [32], as modified by Shertzer [33]. For determination of GSH and oxidized glutathione (GSSG), rinsed cells in suspension were precipitated with 0.1 vol. 50% trichloroacetic acid, followed by spectrophotofluorometric quantitation [34]. Lipid peroxidation decomposition products were determined in cultured cells. Cells were removed from incubation flasks by scraping, followed by centrifugation at 3000 *g* to pellet both live cells and dead cells that had detached previously from the flask. Cells were washed twice in buffer (0.9% NaCl, 50 mM potassium phosphate, pH 7.4), and resuspended in 0.5 mL buffer, followed by the addition of 10 μL of 0.2 M butylated hydroxytoluene in DMSO, 50 μL of 50% (w/v) trichloroacetic acid and 0.3 mL of 1% 2-thiobarbituric acid in 0.28% (w/v) NaOH. The mixture was heated at 100° for 10 min and thiobarbiturate-reactive products were quantified by $A_{532\ nm} - A_{600\ nm}$ as previously described [35].

Statistics. Statistical differences between group mean values were determined by a one-way ANOVA, followed by the Student–Newman–Keuls test for a pairwise comparison of means, using SigmaStat Statistical Analysis software (Jandel Corp.).

RESULTS

Effect of DHII on susceptibility to menadione toxicity. Preincubation with 25 μM DHII produced a time-dependent increase in resistance to menadione in *14CoS/14CoS* cells, with a maximum effect at 24 hr (Fig. 1). The protective effect of DHII in *ch/ch* cells was minimal, with a slight protection observed at 48 hr. The error bars indicated in the lower panel of Fig. 1 were calculated from individual *pairs* of data. These errors better reflect differences between DHII-treated and vehicle-treated cells than the unpaired data presented in the upper two panels, because individual variability in experimental conditions is taken into account. To establish whether pharmacokinetic considerations should be applied to the analysis of the protective effects of DHII, cellular concentrations of DHII were determined. Within 30 min after addition to cells, DHII attained a concentration of 15 ± 4 nmol/mg protein. This value was independent of cell type and remained constant through 48 hr (data not shown). Furthermore, in DHII-treated cells no lipid peroxidation was observed, even in dead and dying cells. This is in contrast to vehicle-treated cells, where cellular lipid peroxidation occurred proportionally to the percentage of dead cells (data not shown). The concentration-dependency for 2-hr menadione toxicity was determined after a 24-hr exposure to DHII, the preincubation period for maximal protection. At every menadione concentration, *14CoS/14CoS* were more resistant than *ch/ch* cells (Fig. 2). Pretreatment with DHII for 24 hr increased the resistance of *14CoS/14CoS* cells to menadione toxicity, while having no effect in *ch/ch* cells.

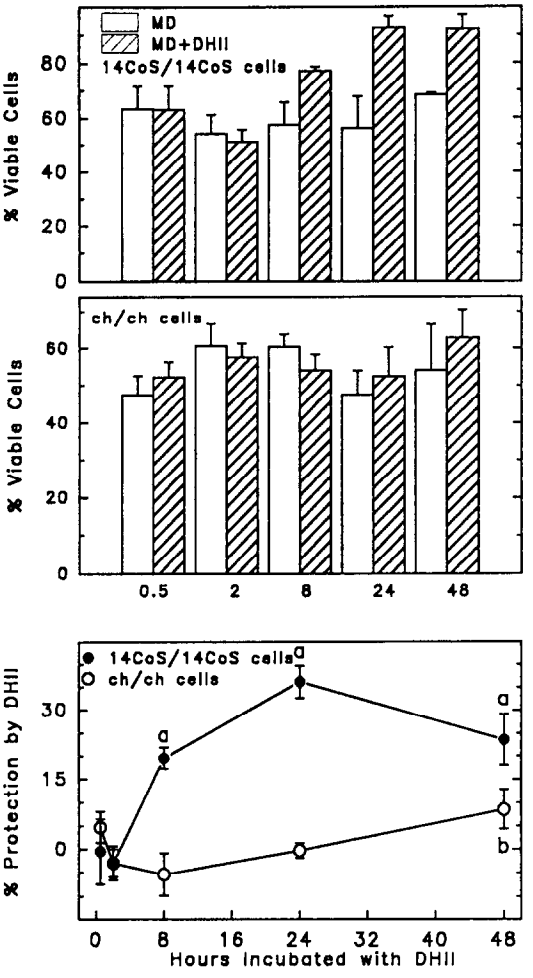

Fig. 1. Effect of duration of cell pretreatment with dihydroindenoindole (DHII), on viability after addition of menadione (MD). *14CoS/14CoS* cells (top panel and closed circles bottom panel) or *ch/ch* cells (middle panel and open circles bottom panel) were pretreated at zero time with DMSO, or a solution of DHII in DMSO vehicle, such that the final concentrations were 0.25% DMSO and 25 μM DHII. At the time after pretreatment indicated in the figure, cells were washed with fresh medium containing either DMSO or a solution of MD in DMSO (0.25%, DMSO concentration). The MD concentrations used were 40 μM (*14CoS/14CoS* cells) or 20 μM (*ch/ch* cells). Cell viability was assessed after 2 hr as a percentage of attached cells after MD treatment, compared with the number of cells in DMSO-treated cultures, in the presence or absence of DHII. Values are means ± SD for four experiments. Percent protection (lower panel) is the mean of the differences in percent cell viability after the addition of MD in the absence or presence of DHII ± SD. Key: (a) significantly greater than zero and greater than the mean value for vehicle-treated controls at $P < 0.05$; and (b) significantly greater than zero at $P < 0.05$.

Enzyme activities and glutathione levels in 14CoS/14Cos *and* ch/ch *cells.* We assayed the two cell lines for a number of redox-associated and related enzyme activities, as well as glutathione concentrations (Table 1). NMO1 activity was about 10-fold higher in *14CoS/14CoS* than in *ch/ch* cells; DHII increased

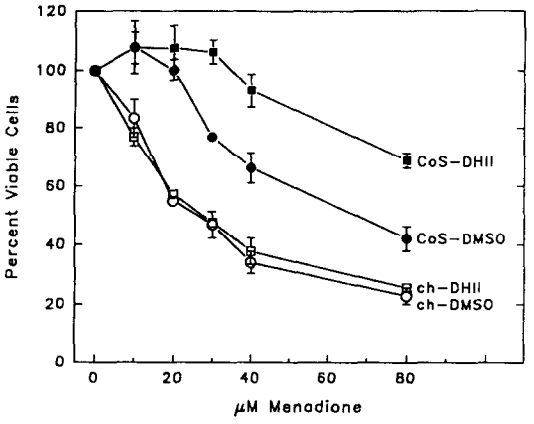

Fig. 2. Effect of dihydroindenoindole (DHII) pretreatment on cell viability after menadione (MD) addition. *14CoS/14CoS* cells (closed symbols) or *ch/ch* cells (open symbols) were treated at zero time with DMSO (circles), or a solution of DHII in DMSO (squares), such that the final concentrations were 0.25% DMSO and 25 μM DHII. After 24 hr, cells were washed and received fresh medium containing either DMSO or a solution of MD in DMSO, with final concentrations of 0.25% DMSO or the MD concentration indicated. Cell viability was assessed as a percentage of attached cells after MD treatment, compared with the number of cells in DMSO-treated cultures. Values are means ± SD for four experiments. The lack of an error bar indicates that the magnitude of error lies within the symbol.

the difference to almost 30-fold, since inductions were about 700 and 280% in *14CoS/14CoS* and *ch/ch* cells, respectively. Of the other enzymes examined, only UGT1 *06 was increased (68%) in *ch/ch* cells. However, in *14CoS/14CoS* cells, activities of GST1A1 (79%), UGT1*06 (98%), and glutathione reductase (101%) were increased significantly. The temporal patterns of induction by DHII for NMO1, glutathione reductase and GST1A1 were similar (Fig. 3), with maximum induction observed at 24 hr after DHII addition, and decreases in activity up to 48 hr. This is similar to the temporal pattern described for DHII-mediated protection from menadione toxicity (Fig. 1, lower panel). GSH concentrations were strongly up-regulated by 24-hr pretreatment with DHII in both *14CoS/14CoS* (134% increase) and *ch/ch* (244% increase) cells (Table 1). However, since the two cell types had very different levels of GSH in untreated cells, the maximal levels achieved in *ch/ch* cells were the same as those in untreated *14CoS/14CoS* cells. Levels of GSH also increased to maximal values 24 hr after DHII administration to both cell types (Fig. 4). However, in contrast to enzyme activities, maximal GSH levels remained constant until at least 48 hr.

DHII-modulated menadione-mediated superoxide formation in the two cell lines. Menadione stimulated the production of superoxide in both cell types in a concentration-dependent fashion (Fig. 5). At every menadione concentration, the rate of superoxide formation was somewhat less in *14CoS/14CoS* cells than in *ch/ch* cells. DHII (5–100 μM) had no immediate effect on superoxide formation in either

Table 1. Enzyme activities and glutathione levels in *14CoS/14CoS* and *ch/ch* cells

Parameter	*14CoS/14CoS* (control)	*14CoS/14CoS* (DHII)	*ch/ch* (control)	*ch/ch* (DHII)
NMO1	22.2 ± 1.7* (7)	157 ± 33*,† (7)	1.98 ± 1.41 (7)	5.56 ± 2.30† (6)
GST1A1	70.3 ± 3.4* (7)	126 ± 35*,† (7)	48.9 ± 3.9 (7)	52.3 ± 11.9 (7)
UGT1*06	0.59 ± 0.13* (4)	1.17 ± 0.31*,† (4)	0.28 ± 0.02 (4)	0.47 ± 0.06† (4)
GSHpx	35.3 ± 7.0 (4)	31.6 ± 2.2 (4)	32.3 ± 1.7 (4)	37.0 ± 6.9 (4)
GSSGred	84.0 ± 27.0* (7)	169 ± 28† (7)	157 ± 30 (7)	165 ± 29 (7)
CYP1A1	ND‡	ND	ND	ND
CYP1A2	ND	ND	ND	ND
SOD	42.1 ± 7.9* (4)	42.3 ± 0.7* (4)	31.1 ± 3 0 (4)	33.4 ± 6.8 (4)
NADPH *c* red	6.19 ± 2.12 (8)	4.87 ± 2.1 (8)	6.93 ± 1.78 (8)	7.38 ± 1.42 (8)
GSH	16.9 ± 1.0* (7)	39.6 ± 3.9*,† (7)	3.81 ± 0.19 (7)	13.1 ± 0.7† (7)
GSSG	4.80 ± 0.37* (7)	7.58 ± 0.8*,† (7)	1.76 ± 0.1 (7)	3.14 ± 0.23† (7)

14CoS/14CoS cells or *ch/ch* cells were treated at zero time with DMSO, or a solution of DHII in DMSO vehicle, such that the final concentrations were 0.25% DMSO and 25 μM DHII. After 24 hr, each group was assayed for the parameters indicated (NMO1, NAD(P)H:menadione oxidoreductase; GST1A1, glutathione transferase form 1A1; UGT1*06, UDP glucuronosyltransferase form 1*06; GSHpx, glutathione peroxidase; GSSGred, glutathione reductase; CYP1A1 and 1A2, cytochromes P450 forms 1A1 and 1A2; SOD, Cu,Zn-superoxide dismutase; NADPH *c* red, NADPH cytochrome *c* oxidoreductase). Specific activities are expressed as mean values ± SD (number of independent determinations). Enzyme values are nmol/min/mg protein, except for SOD (expressed as units of activity/mg protein, defined as the inverse of the mg protein required to inhibit by 50% the rate of reduction of nitroblue tetrazolium dye by xanthine/xanthine oxidase-generated superoxide [30]). GSH and GSSG levels are expressed as mean nmol/mg protein. CYP1A1 and CYP1A2 activities were assayed as ethoxyresorufin *O*-deethylase (fluorescence units/min/mg protein) and acetanilide 4-hydroxylase (units/mg protein), respectively.

* Mean values in *14CoS/14CoS* cells were significantly different from those in *ch/ch* cells, treated in the same manner, at the confidence level of $P < 0.05$.

† Mean values for DHII-treated cells were significantly different from vehicle-treated controls at the confidence level of $P < 0.05$.

‡ ND, not detectable.

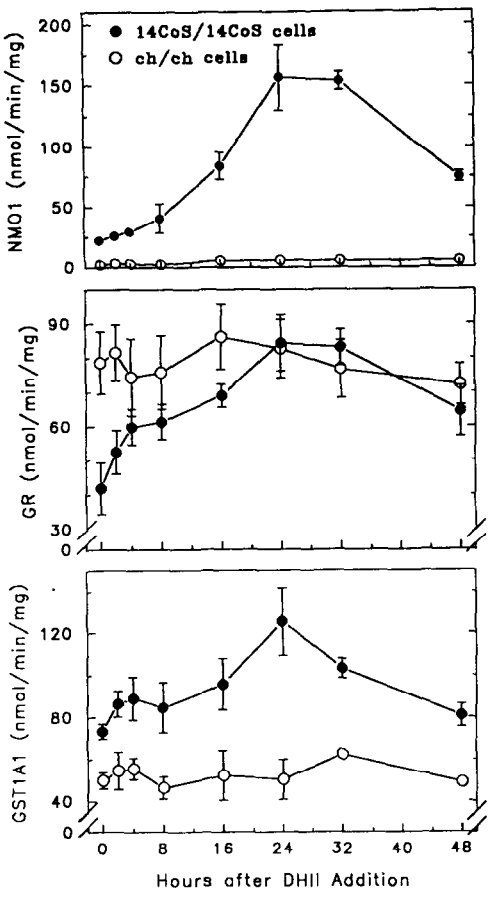

Fig. 3. Effect of time of incubation with dihydroindenoindole (DHII) on enzyme activities. *14CoS/14CoS* cells or *ch/ch* cells were treated at zero time with DMSO, or a solution of DHII in DMSO vehicle, such that the final concentrations were 0.25% DMSO and 25 μM DHII. At the time after treatment indicated in the figure, each group was assayed for activities of NMO1 (quinone reductase), GSSG reductase (GR), and glutathione *S*-transferase (GST1A1), as described in Methods. DMSO had no effect on enzyme activities. The data for the effect of DHII on enzyme activities are expressed as means ± SD for four experiments. The lack of an error bar indicates that the magnitude of error lies within the symbol.

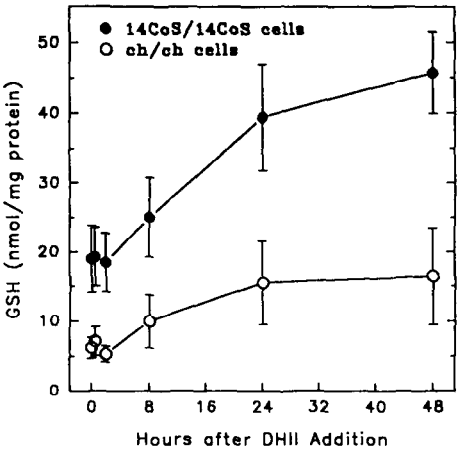

Fig. 4. Effect of incubation time with dihydroindenoindole (DHII) on glutathione (GSH) levels. *14CoS/14CoS* cells or *ch/ch* cells were treated with DMSO or 25 μM DHII as described in the legend to Fig. 3. At the time after treatment indicated in the figure, cells were assayed for GSH levels as described in Methods. DMSO did not affect GSH levels. The data following DHII treatment are shown as mean ± SD for four experiments.

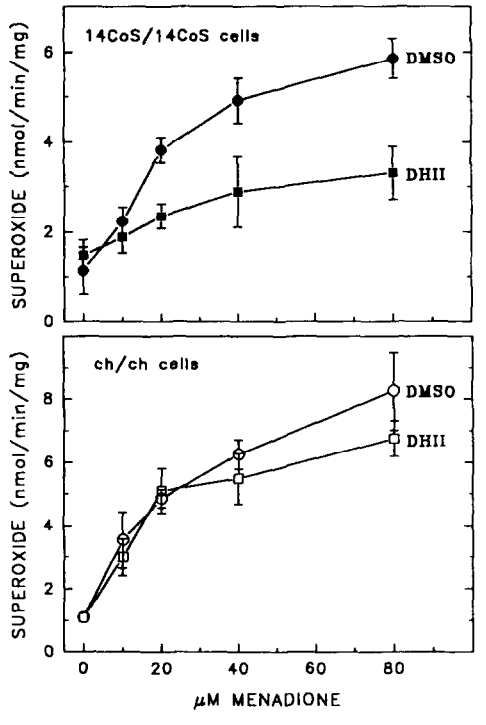

Fig. 5. Menadione (MD)-mediated superoxide formation in control and dihydroindenoindole (DHII)-treated cells. *14CoS/14CoS* cells (closed symbols) or *ch/ch* cells (open symbols) were treated at zero time with DMSO (circles), or a solution of DHII in DMSO (squares), such that the final concentrations were 0.25% DMSO and 25 μM DHII. After 24 hr, each treatment group was assayed for the rate of MD-mediated superoxide formation, expressed as nmol cytochrome *c* reduced/min/mg protein. MD was added as a solution in DMSO, with the DMSO concentration held constant at 0.25%. Values are means ± SD for four experiments. The lack of an error bar indicates that the magnitude of error lies within the symbol.

cell type (data not shown). However, preincubation of *14CoS/14CoS* cells with DHII produced an approximate 50% decrease in the rate of menadione-generated superoxide production; in contrast, DHII preincubation had no effect on superoxide formation in *ch/ch* cells.

DHII-modulated menadione effects on intracellular GSH and GSSG concentrations. Menadione produced a concentration-dependent diminution in the levels of GSH in both cell types (Fig. 6). This effect was dependent on the initial concentration of GSH, with the strongest GSH depletion (91%) observed in untreated *ch/ch* cells, intermediate effects in DHII-pretreated *ch/ch* cells (79%) and untreated *14CoS/14CoS* cells (69%), and the smallest effects in DHII-pretreated *14CoS/14CoS* cells (29%). The

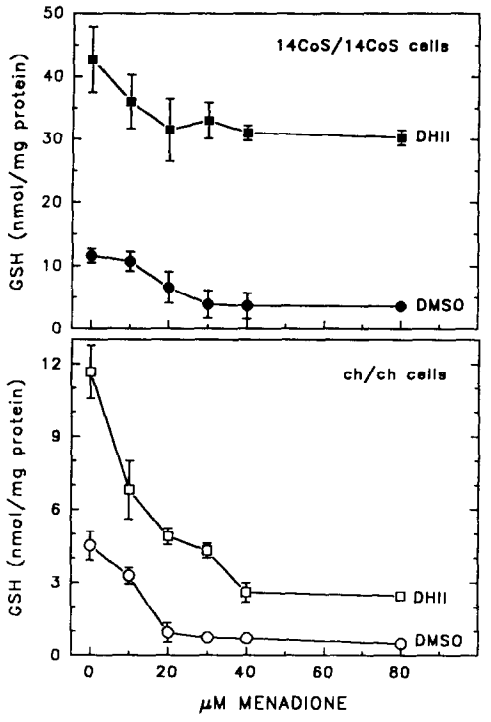

Fig. 6. Levels of GSH following treatment with dihydroindenoindole (DHII) and menadione (MD) in the two cell lines. *14CoS/14CoS* cells (closed symbols) or *ch/ch* cells (open symbols) were treated at zero time with DMSO (circles), or a solution of DHII in DMSO (squares), such that the final concentrations were 0.25% DMSO and 25 μM DHII. After 24 hr, each treatment group received either DMSO or a solution of MD in DMSO, with final concentrations of 0.25% DMSO or the menadione concentration indicated. After 2 hr, GSH levels were determined as described in Methods. DMSO did not affect GSH levels. Values for the DHII treatment groups are means ± SD for four experiments. The lack of an error bar indicates that the magnitude of error lies within the symbol.

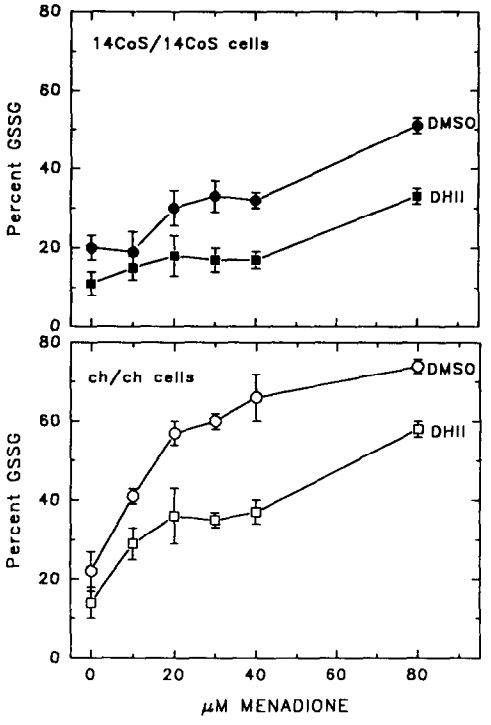

Fig. 7. Percent glutathione as GSSG following treatment with dihydroindenoindole (DHII) and menadione in the two cell lines. Cells were treated as described in the legend to Fig. 6. Values for the DHII treatment groups are expressed as mean percent oxidized glutathione (GSSG) ± SD for four separate experiments. See Table 1 for GSH and GSSG control values.

percentage of GSSG is a useful indicator of conditions of cellular oxidative stress. Menadione generated a concentration-dependent increase in percent GSSG in both cell types, with *14CoS/14CoS* cells displaying much greater resistance to menadione-mediated oxidative stress than *ch/ch* cells (Fig. 7). Pretreatment with DHII for 24 hr diminished this oxidative stress response in both cell types, with a significantly more pronounced inhibition in *14CoS/14CoS* cells. The inhibition of menadione-mediated increase in percent GSSG required preincubation with DHII (data not shown).

DISCUSSION

Attempts to decrease adverse effects in humans from toxic, mutagenic or carcinogenic agents have emphasized decreasing concentrations of, or exposure to, such agents. An alternate approach is the development of non-toxic compounds that confer a lower degree of risk associated with human exposure to toxicants and carcinogens. A wide variety of such chemoprotective compounds have been identified [14, 17, 36–39]. Several mechanisms may be responsible for protection, depending on the chemoprotectant, the toxic substance, and the biological system. These mechanisms include scavenging of reactive chemical species and biomembrane stabilization [17, 35, 40], and the modification of enzymatic profiles that could enhance detoxification pathways and/or inhibit activation pathways for toxicants and carcinogens [14, 19, 20, 37, 39, 41]. The indenoindoles are a class of chemoprotectants (including DHII) that act by several mechanisms, including radical scavenging [15–17], membrane stabilization [17, 42], protection against protein thiol depletion [18], and enzyme induction [19, 20, 43]. However, it has not been possible to assign relative importance amongst the possible mechanisms of action eliciting protection; such information would be useful in order to design novel compounds with enhanced efficacy for chemoprotection.

In this report we demonstrate that the protection against menadione toxicity afforded by DHII requires a preincubation period with the protectant. The time course for the development of protection is similar

to that for the induction of NMO1, GST1A1 and glutathione reductase. However, it does not appear that glutathione reductase plays an important role in protection, since the maximal level of induction in the menadione-resistant *14CoS/14CoS* cells only reached the level for the menadione-susceptible untreated *ch/ch* cells. However, GST1A1, UGT1*06 and particularly NMO1 were elevated to significantly greater levels in *14CoS/14CoS* cells relative to *ch/ch* cells. These enzymes have been suggested to be involved in the protection against chemical carcinogenesis afforded by a class of compounds known as Michael Reaction acceptors, which consist of an electron withdrawing moiety (such as a carbonyl group) α–β to an olefinic or acetylenic group [14, 37, 41]. Thus, induction of these enzymes produces a protective enzyme profile that may serve to detoxify and aid in the elimination of menadione and metabolically related chemicals such as other quinones, quinone imines and azo dyes.

It was proposed previously [9] that in untreated cells the greater resistance to oxidative stress of *14CoS/14CoS* cells relative to *ch/ch* cells was mediated by both GSH and the phase II enzymes NMO1, UGT1*06 and GST1A1; it appeared that GSH was the dominant factor. For the DHII-mediated increased protection afforded to *14CoS/14CoS* cells, it appears that both GSH and enzyme induction are again important. However, at 48 hr after DHII treatment, the GSH levels remained elevated in both cell types, and the levels of GSH in DHII-treated *ch/ch* cells approached the level determined for untreated *14CoS/14CoS* cells. However, DHII did not increase menadione resistance in *ch/ch* cells, and the level of protection in *14CoS/14CoS* cells diminished at 48 hr post-treatment. These results suggest that for DHII-mediated protection, enzyme induction is the dominating mechanism. This conclusion is strengthened by the observation that DHII did not have a direct effect on superoxide production or scavenging, and that the levels of DHII did not change throughout the course of the pretreatment; such levels were sufficient to prevent lipid peroxidation from occurring at any time after treatment with menadione.

We have shown previously that in mouse and rat liver, as well as cultured hepatoma wild type cells (Hepa-1c1c7), DHII induced enzymes of the [*Ah*] gene battery, namely CYP1A1, NMO1, UGT1*06, and GST1A1 [19, 20, 43]. In hepa-1 cells, this induction appeared to be mediated by the ability of DHII to interact directly with a functional [*Ah*] receptor, but not the functional gene product CYP1A1. In contrast, DHII induced higher levels of GSH only in [*Ah*] competent cells, and not in [*Ah*]-defective mutant cells, suggesting that CYP1A1 is required to convert DHII to a metabolite that, in turn, mediates GSH enhancement. Previous studies have shown that the concentration of GSH in cells is determined primarily by the activity of the allosterically controlled rate-limiting enzyme in its biosynthetic pathway, namely γ-glutamylcysteine synthetase [44, 45]. The possible mechanisms by which chemicals might enhance GSH levels in cells or tissues were reviewed recently [46]. Allosteric up-regulation of the activity of γ-glutamylcysteine synthetase normally involves a change in GSH or GSSG pools, which does not occur with DHII treatment. The more likely possibility is transcriptional and translational up-regulation of enzyme synthesis. Such regulation has been reported for compounds with an α–β-unsaturated carbonyl group [47]. The putative metabolite of DHII that might have similar induction properties has not been identified.

We conclude from this study that DHII exerts a protective effect from menadione toxicity in *14CoS/14CoS* cells by virtue of inducing a protective enzyme profile that promotes the detoxification and excretion of the toxicant. By acting via such a mechanism, it would be expected that DHII would protect similarly against other chemicals that are metabolized by similar pathways, namely other quinones, quinone imines and azo dyes. Induction of higher levels of GSH, and the direct antioxidant and membrane stabilizing properties of DHII, may also serve as an important mechanism for protection against other toxicants or carcinogens. The lack of protection against menadione toxicity and poor enzyme induction properties of DHII in *ch/ch* cells may be peculiar to this line of immortalized cells, or it may reflect a true heterogeneity of various cell types *in vivo* to respond to this class of compound. The *14CoS/14CoS* and *ch/ch* cell lines provide an important model system to investigate these possibilities.

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