Pergamon

Biochemical Pharmacology, Vol. 50, No. 11, pp. 1885–1891, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)02083-7

RESPONSE OF [*Ah*] BATTERY GENES TO COMPOUNDS THAT PROTECT AGAINST MENADIONE TOXICITY

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(*Received* 24 *April* 1995; *accepted* 14 *August* 1995)

Abstract—We have studied the response of genes in the dioxin-inducible [*Ah*] battery to three compounds that protect mouse hepatoma cells (Hepa-1c7c7 wild-type, *wt*) against menadione toxicity. Pretreatment of *wt* cells with 25 μM 5,10-dihydroindeno[1,2-*b*]indole (DHII), 25 μM *tert*-butylhydroquinone (tBHQ), or 10 μM menadione itself, generated substantial protection against toxicity produced by subsequent menadione exposure. The gene response was examined in *wt* cells, and three mutant lines: CYP1A1 metabolism-deficient (*c37* or P_7^-); nuclear translocation-impaired (*c4* or nt^-); and AHR-deficient (*c2* or r^-, containing <10% of normal functional receptor levels). DHII treatment of *wt* cells for 12 hr markedly elevated the enzyme activities and mRNA levels of genes in the [*Ah*] battery: aryl hydrocarbon hydroxylase (*Cyp1a1*), NAD(P)H:menadione oxidoreductase (*Nmo1*), cytosolic aldehyde dehydrogenase class 3 (Ahd4), and UDP-glucuronosyltransferase form 1*06 (*Ugt1*06*). Treatment of the *c4* and *c2* cells with DHII failed to induce mRNA levels of the genes, indicating that induction of the [*Ah*] gene battery by DHII is aromatic hydrocarbon receptor (AHR)-mediated. On the other hand, neither tBHQ nor menadione caused increases in CYP1A1 mRNA, but tBHQ significantly enhanced the NMO1, AHD4, and UGT1*06 mRNA levels in all three mutant cell lines. In conclusion, we expect one or more putative electrophile response elements (EpRE), previously found in the regulatory regions of the murine *Nmo1*, *Ahd4*, and *Ugt1*06* genes, to be functional in responding to phenolic antioxidants.

Key words; [*Ah*] gene battery; aromatic hydrocarbon response element; dihydroindenoindole; gene regulation; electrophile response element; enzyme induction; hepatoma cells; menadione; TCDD

The murine [*Ah*] gene battery comprises at least six genes that are coordinately induced by TCDD§ and polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene. In addition to two Phase I cytochrome P450 genes, *Cyp1a1* and *Cyp1a2*, there are four Phase II genes: *Nmo1; Ahd4; Ugt1*06;* and a glutathione transferase having 2,4-dinitro-1-chlorobenzene as substrate (GST Ya, Gsta1) [1–3]. Induction by TCDD is mediated by a soluble intracellular protein, the AHR. Following binding to a ligand such as TCDD, the AHR forms a heterodimer with the aromatic receptor nuclear translocator (ARNT), translocates into the nucleus, binds to the AhRE (also termed xenobiotic or dioxin response elements, XREs, DREs), and turns on transcription of the [*Ah*] battery genes [reviewed in refs. 3–6]. These AhREs have been identified in the 5′ regulatory domain of all six [*Ah*] battery genes.

Recent studies have shown that GSTA1 and NMO1 enzyme activities or mRNA levels can be increased by certain electrophilic compounds such as quinones, coumarins, and other compounds containing an olefinic bond in conjugation with an electron-withdrawing moiety (i.e. Michael Reaction acceptor molecules), or compounds easily oxidized into such compounds [7, 8]. Therefore, it has been suggested that the inducible expression of these two genes is controlled by an EpRE (also termed antioxidant response element, ARE) that is activated by binding to an electrophile-sensitive or reactive oxygen-sensitive EpRE binding protein(s) [9–11]. Thus far in published reports, EpREs have been identified in the upstream regions of the rat, murine, and human *NMO1* genes, and the rat and mouse *GSTA1* genes, the murine *Ahd4* and *Ugt*06* [12–17].

We have studied genes of the [*Ah*] battery in response to three structurally diverse compounds: 5,10-dihydroindeno[1,2-*b*]indole (DHII), *tert*-butylhydroquinone (tBHQ), and menadione. Interestingly, each of these compounds protects mouse hepatoma cells against toxicity from subsequent exposure to menadione. DHII has been shown previously to increase certain [*Ah*] battery enzyme activities in cultured mouse cells [18]. This study was undertaken to extend our understanding of the expression of [*Ah*] battery genes involved in modulating susceptibility to toxicants such as menadione.

To determine AHR-dependent vs -independent mechanisms of induction, this laboratory has routinely used the mouse hepatoma Hepa-1c7c7 wild-type (*wt*) parent

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§ Abbreviations: AHR, aromatic hydrocarbon receptor; AhRE, aromatic hydrocarbon response element; DHII, 5, 10-dihydroindeno[1,2-*b*]indole; EpRE, electrophile response element; EDTA, ethylenediaminetetraacetic acid; tBHQ, *tert*-butyl hydroquinone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; [*Ah*] gene battery: *Ahd4*, cytosolic aldehyde dehydrogenase class 3; Gsta1, glutathione S-transferase (Ya or class α); *Nmo1*, NAD(P)H:menadione oxidoreductase, [NAD(P)H:quinone acceptor oxidoreductase, azo dye reductase, quinone reductase, DT-diaphorase]; *Ugt1*06*, uridine diphosphoglucuronic acid glucuronosyltransferase form 1*06. By convention, murine genes are denoted by italicized lower case letters and rat genes by italicized capital letters, whereas the mRNA or enzyme (gene product) are denoted by capital letters that are not in italics. In the case of Ahd4, the mRNA and enzyme are also referred to as ALDH3c.

line and three mutant lines: *c37*, CYP1A1 metabolism-deficient (P_1^-); *c4*, nuclear translocation-impaired (nt^-), lacking the Ah receptor nuclear translocator (ARNT); and *c2*, AHR-deficient (r^-) containing <10% of normal functional receptor levels. CYP1A2 expression does not occur in *wt* cells, and therefore has not been studied. Our results show that the [*Ah*] Phase II genes are inducible by tBHQ and menadione in an AHR-independent pathway, whereas the DHII induction occurs via the AHR.

MATERIALS AND METHODS

Chemicals

All chemicals, reagents, and enzymes used in this study were obtained from either Sigma Chemical Company (St. Louis, MO, U.S.A.) or from Aldrich Chemical Company (Milwaukee, WI, U.S.A.), except as noted below. DHII was synthesized as previously described [19].

Cell culture conditions

The *wt* mouse hepatoma Hepa-1 cells, the ARNT-defective *c4*, the metabolism-defective *c37*, and the receptorless *c2* [20] were generous gifts of O. Hankinson (UCLA, Los Angeles, CA, U.S.A.). All cell lines were routinely grown in modified Eagle's α-medium containing 5% fetal calf serum. When required, TCDD treatment (final concentration of 20 nM, *p*-dioxane vehicle) was carried out for 12–24 hr, or benzo[*a*]pyrene treatment (final concentration of 10 μM, DMSO vehicle) was carried out for 6–48 hr. Treatment was carried out for the 6–24 hr with final concentrations of DHII (25 or 50 μM) or tBHQ (25 μM), or menadione (1, 10, and 30 μM); the compounds were first dissolved in DMSO at 1000 times the final concentrations. The concentration of the vehicles in the treated cells did not exceed 0.1%, and the untreated experimental controls were treated with 0.1% of the corresponding vehicle; no effect on the cell viability was seen at these concentrations of vehicles. Cell viability was assessed by rinsing the attached cells with buffer consisting of 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).

Preparation of subcellular fractions

After being rinsed twice with ice-cold phosphate-buffered saline, the cells were scraped from the tissue culture flasks. The harvested suspension was centrifuged at 1,500 *g* for 5 min, and the cell pellet was resuspended in the homogenization buffer (0.1 M sodium pryophosphate, pH 8.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol). Typically, one culture flask of cells was resuspended in 1 mL of buffer. The cell suspensions were sonicated in ice, with 3 periods of 10 sec interrupted by two intervals of the same duration, to avoid overheating. The cell-free suspension was centrifuged at 3,000 *g* for 10 min, and the supernatant fraction was centrifuged at 105,000 *g* for 1 hr. The soluble fraction was used for the ALDH3c and NMO1 assays, and the microsomal pellet was resuspended in 0.3 mL of homogenization buffer and used for the CYP1A1 and UGT1*06 assays.

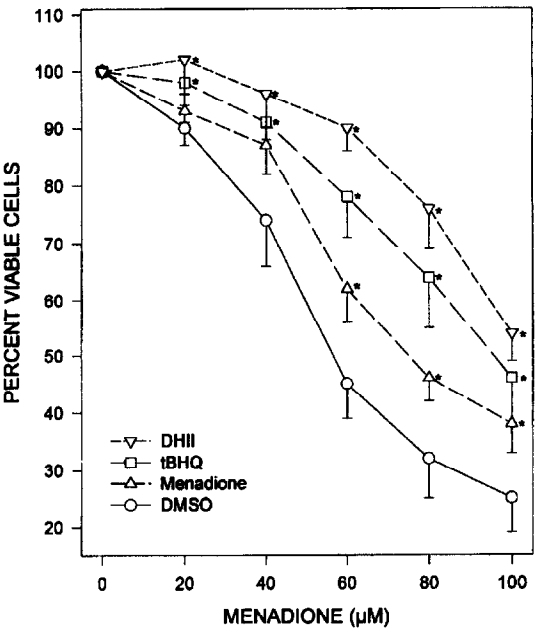

Fig. 1. Effect of menadione pretreatment on menadione toxicity in Hepa-1 *wt* cells. Cells were treated with DMSO (circles) or solutions of either DHII (down triangles), tBHQ (squares), or menadione (up triangles) in DMSO, such that the final concentrations were 0.1% DMSO and either 25 μM DHII, 25 μM tBHQ, or 10 μM menadione. After 24 hr, cells were washed and received fresh medium containing either DMSO or a solution of menadione in DMSO, with final concentrations of 0.25% DMSO or the menadione concentration indicated. Cell viability was assessed 2 hr after the final additions, as the percentage of attached cells after menadione treatment relative to the number of attached cells in the absence of menadione. The results are expressed as mean values ± SD (n = 3), with asterisks inside the symbol indicating a significantly different mean value ($P < 0.05$) than that obtained using cells treated with DMSO alone.

Enzyme assays

The activities of NMO1 (DT-diaphorase) [EC 1.6.99.2] [21], CYP1A1 as ethoxyresorufin O-deeth-

Table 1. Effect of DHII and tBHQ on [*Ah*] battery enzyme activities in *wt* cells

Enzyme	Control (DMSO)	DHII	tBHQ
NMO1	130 ± 10	480 ± 69*	410 ± 49*
ALDH3c	1.8 ± 0.2	28 ± 0.9*	5.9 ± 0.4*
UGT1*06	2.0 ± 0.3	3.8 ± 0.8*	3.2 ± 0.6*
CYP1A1	<0.01	0.38 ± 0.04*	<0.01

Cells were treated at zero time with DMSO, or a solution of DHII or tBHQ in DMSO vehicle, such that the final concentrations were 0.25% DMSO, 25 μM DHII, or 25 μM tBHQ. After 24 hr, each group was assayed for the parameters indicated. Specific activities are expressed as mean values ± SD for four independent determinations. Values are nmol/min/mg protein, except for CYP1A1 activity, which was assayed as fluorescence units/min mg protein for ethoxyresorufin O-deethylase.

* Mean values for DHI- or tBHQ-treated cells are significantly different from DMSO vehicle treated controls at the confidence level of $P < 0.001$.

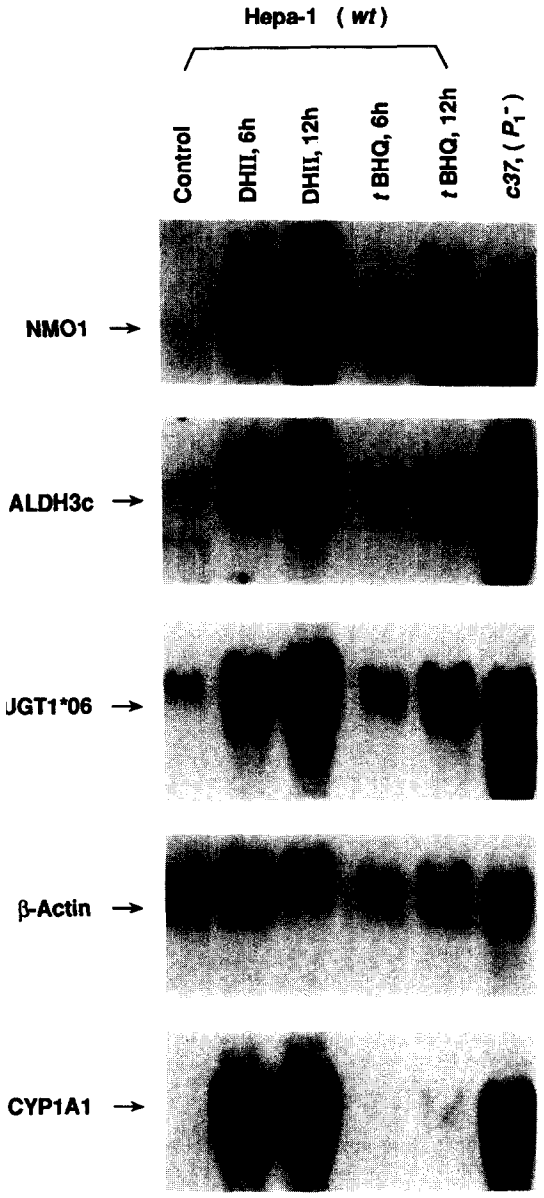

Fig. 2. Northern hybridization analysis of NMO1, ALDH3c, UGT1*06, and CYP1A1 mRNA levels in control, DHII-treated, and tBHQ-treated Hepa-1 *wt* cells. The CYP1A1 metabolism-deficient (P_1^-) cell line *c37* (right lane) is shown as a positive control in which all three Phase II mRNAs are elevated due to an endogenous "oxidative stress" response. The β-actin mRNA is a control to assess the amount RNA loaded in each lane.

ylase [microsomal cytochrome P_1450; aryl hydrocarbon hydroxylase] [22], and cytosolic ALDH3c [2] were assayed as described in the references cited. Microsomal UGT1*06 (UDP glucuronosyltransferase) [EC 2.4.1.17] was assayed using *p*-nitrophenol as substrate [23], as modified by Shertzer [24]. Protein was measured by the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL), according to details supplied by the manufacturer. Specific activities are expressed in units/mg protein.

RNA extraction and Northern blots

RNA was extracted by the acid guanidinium thiocyanate method [25]. Total RNA (10 μg) was separated in formaldehyde-agarose gels and transferred to Nytran. Transfers were carried out for 2 to 4 hr with the use of a semi-dry blotting apparatus (JKA BioTech, Copenhagen, Denmark) at <0.8 mA per cm^2 of gel surface. Prehybridizations and hybridizations were carried out at 42° in a solution containing 50% deionized formamide, 6X SSC (SSC = 0.9 M NaCl and 0.09 M sodium citrate, pH 7.0), 2.5X Denhardt's solution [0.5 g Ficoll/L, 0.5 g of poly(vinylpyrrolidone)/L, 0.5 g of bovine serum albumin/L], 0.5% sodium dodecylsufate (SDS), and denatured salmon sperm DNA (0.1 mg/mL). Radioactivity labeled probes were prepared by random priming [26], using [α-^{32}P]dCTP (3,000 Ci/mmole, New England Nuclear/DuPont) as the labeled precursor, and were added to the hybridization solutions at 5×10^6 to 10×10^6 cpm/mL. After hybridization for 16–20 hr, the filters were washed twice in 2X SSC and 0.1% SDS for 10 min at room temperature, and then twice in 0.1X SSC and 0.1% SDS for 30 min at 50°. The filters were then exposed for 48 hr to Kodak XAR-5 film at −70° with intensifying screens. Probes included the murine AHD4 [3], UGT1*06 [16] and NMO1 [27] cDNAs, and the chicken β-actin cDNA [28].

Statistics

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

RESULTS AND DISCUSSION

The murine [*Ah*] gene battery consists of at least six genes (*Cyp1a1, Cyp1a2, Nmo1, Ahd4, Ugt1*06,* and *Gsta 1*) that share the common feature of being up-regulated by AHR agonists, and down-regulated by a functional CYP1A1/1A2, enzyme [4]. To date, we have identified three distinct regulatory mechanisms for the [*Ah*] gene battery: (a) The AHR-ARNT heterodimer, acting through AhREs, is a positive regulator of all six [*Ah*] battery genes; (b) a chromosome 7-mediated pathway up-regulates the Phase II genes (acting via the EpRE), but not the Phase I genes; and (c) a CYP1A1/CYP1A2 metabolism-dependent repression acts possibly via a

Table 2. Effect of DHII and tBHQ on [*Ah*] battery enzyme activities of *c4* (*nt*) cells

Enzyme	Control	DHII	tBHQ
NMO1	107 ± 9	140 ± 39	220 ± 23*
ALDH3c	0.17 ± 0.3	0.20 ± 0.3	15 ± 3.6*
UGT1*06	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.2*
CYP1A1	<0.01	<0.01	<0.01

Cells were treated with DMSO, DHII, or tBHQ, and assayed for NMO1, ALDH3c, UGT1*06, and CYP1A1 as described in the legend to Table 1. Specific activities are expressed as mean values ± SD for four independent determinations. Values are nmol/min/mg protein, except for CYP1A1 activity, which was not detectable as assayed (fluorescence units/min for ethoxyresorufin O-deethylase.

* Mean values for DHII- or tBHQ-treated cells are significantly different from DMSO vehicle treated controls at the confidence level of $P < 0.001$.

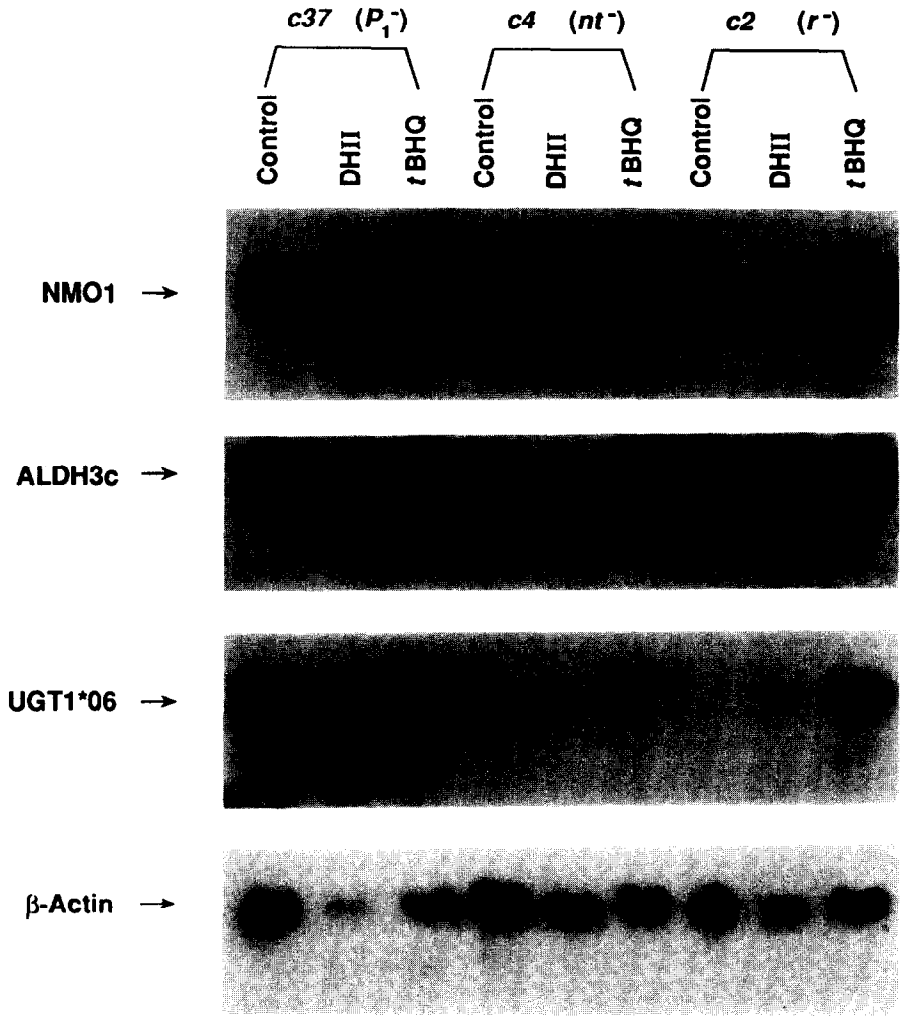

Fig. 3. Northern hybridization analysis of NMO1, ALDH3c, and UGT1*06 mRNA levels in control, DHII-treated, and tBHQ-treated *c37*, c4, and c2 mutant cell lines. The β-actin mRNA is a control to evaluate the amount of RNA loaded in each lane.

negative response element (NRE) [reviewed in 3, 4]. In this study we have examined the effects of an AHR agonist aromatic heterocycle (DHII), a potent antioxidant hydroquinone (tBHQ), and a redox-active electrophilic quinone (menadione itself) on protection against menadione toxicity, and on the enzyme activities and mRNA levels of the murine [*Ah*] battery genes.

DHII is a synthetic aromatic indolic heterocyclic antioxidant [29]. Previous studies have shown that DHII protects *in vivo* and *in vitro* against hepatotoxicity resulting from exposure to a variety of toxicants, including carbon tetrachloride, N-methyl-N′-nitro-N-nitrosoguanidine, methyl methanesulfonate, and menadione [19, 30–33]. The DHII-mediated chemoprotection appeared to be correlated with the induction of certain Phase II drug-metabolizing enzymes including NMO1 and GST [31, 32], and also with an enhancement of GSH levels in cultured c^{14CoS}/C^{14CoS} hepatocytes [18]. However, the genetic mechanism for DHII induction has not previously been evaluated.

Effect of DHII, tBHQ, or menadione on susceptibility to menadione toxicity

Pretreatment of *wt* cells for 24 hr with 25 μM DHII, 25 μM tBHQ, or 10 μM menadione protected against subsequent exposure to higher concentrations of menadione (Fig. 1). The order of effectiveness for protection from menadione toxicity was DHII > tBHQ > menadione.

DHII-mediated induction is AHR-dependent

Treatment of *wt* cells with 25 μM DHII also produced marked increases in the activities of CYP1A1, ALDH3c (15-fold), NMO1 (3-fold), and significant increases in the activities of UGT1*06 (92%) (Table 1). In addition, treatment of *wt* cells with DHII for 6 or 12 hr markedly increased the mRNA levels of NMO1, ALDH3c, UGT1*06, and CYP1A1 (Fig. 2). On the contrary, DHII treatment did not affect these enzyme activities (Table 2) or mRNA levels (Fig. 3) in the *c4* mutant line. It is noteworthy that the expression of ALDH3c and UGT1*06 is lower in untreated *c4* cells compared to *wt* cells, indicating that the AHR-ARNT heterodimer might be involved in the basal expression of these genes.

As had been previously shown, the basal NMO1, ALDH3c, and UGT1*06 mRNA levels are substantially augmented in *c37* cells, due to an absence of functional CYP1A1 protein [2, 34, 35]. Such substantial derepression results in the near maximal expression of these

genes, such that inducing agents produce minor or no further induction. In some instances (e.g. Fig. 4) we observed two RNA bands hybridizing with the UGT1*06 probe; it is likely due to multiple polyadenylation sites found in the UGT1*06 cDNA [16]. We also found small increases in DHII-induced NMO1, ALHD3c, and UGT1*06 mRNA levels in the *c2* mutant line (Fig. 3), which is known to contain a small amount of functional AHR [36].

We found that treatment of *wt* cells with DHII produced marked increases in the activities of Phase I and Phase II enzymes of the [*Ah*] gene battery, as well as the levels of their associated mRNAs; the induction was diminished in *c2* cells having little functional AHR. Conversely, DHII treatment did not affect these parameters in the *c4* mutant line, which lacks the ARNT protein responsible for nuclear translocation of the AHR. These data suggest that DHII transcriptionally activates the [*Ah*] battery genes via an AHR-dependent mechanism.

tBHQ-mediated induction is AHR-independent and requires the EpRE

Treatment of *wt* cells with 50 μM tBHQ for 24 hr produced significant increases in NMO1, ALDH3c, and UGT1*06 enzyme activities, but had no effect on CYP1A1 activity (Table 1). Treatment with tBHQ for 6 and 12 hr also caused significant elevations in NMO1, ALDH3c, and UGT1*06 mRNA levels, but not CYP1A1 mRNA (Fig. 2). Even in *c37* cells, tBHQ treatment further enhanced the already elevated mRNA levels of the *Nmo1*, *Aldh3c*, and *Ugt1*06* genes (Fig. 3).

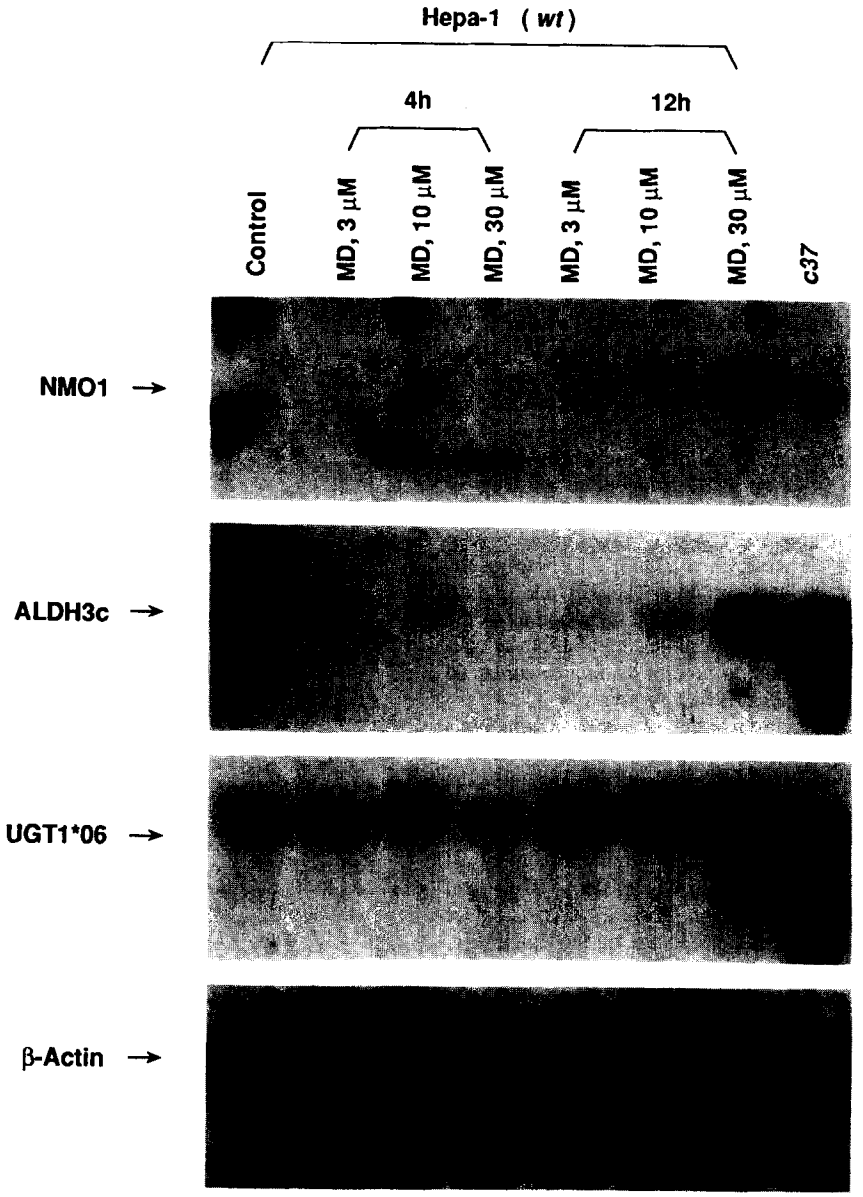

Fig. 4. Northern hybridization analysis of NMO1, ALDH3c, and UGT1*06 mRNA levels in control and menadione-treated *wt* cells. The CYP1A1 metabolism-deficient (P_1^-) cell line *c37* (right lane) is shown as a positive control in which all three Phase II mRNAs are elevated due to putative derepression. The β-actin mRNA is a control to assess the amount of RNA loaded in each lane.

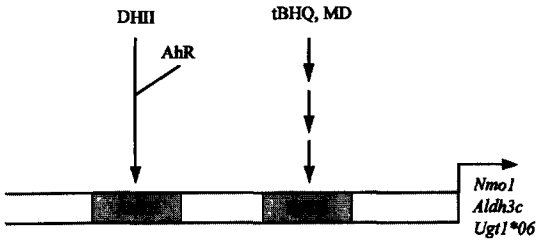

Fig. 5. Diagram of conclusions reached with this study. DHII presumably binds to the Ah receptor (AHR), and the inducer-bound receptor participates in the complex that interacts with the AhREs. Two other compounds, tBHQ and menadione, participate in an unknown number of steps in a signal transduction pathway, which results in activation of the *Nmo1*, *Ahd4*, and *Ugt*06* genes via the EpRE.

Because of this derepression, for the treated cells lower amounts of mRNA (indicated by lower amounts of β-actin) were loaded onto the gel to produce the similar levels of transcripts. In the *c4* mutant line, which lacks the ARNT protein responsible for nuclear translocation of the AHR, constitutive levels of NMO1, ALDH3c, and UGT1*06 mRNA were nil, but were significantly enhanced after tBHQ treatment. The corresponding enzyme activities were also enhanced by tBHQ, consistent with the Northern blot data.

In addition to the results obtained for tBHQ, we found that the potent electrophile menadione markedly induced the mRNA levels of all Phase II [*Ah*] genes (Fig. 4), but not CYP1A1 (negative data not shown), 12 hr after the treatment at near-toxic menadione concentrations. These results suggest that the tBHQ- and menadione-mediated induction processes do not require a functional AHR, but rather operate via the EpRE. Using a synthetic 41-bp oligonucleotide probe, containing sequences between −754 to −713 of the murine *Gsta1* gene 5′-flanking region that corresponds to the EpRE, in gel mobility assays, we have found a DNA-protein complex in both untreated and tBHQ-treated *wt* cells [37]. These results are in agreement with other studies [10, 12], and suggest that a constitutively expressed protein is bound to the EpRE. Treatment with electrophiles could alter the conformation of a *trans*-acting protein leading to enhanced transcription of the [*EpRE*] battery genes.

In conclusion, we found that the three compounds used in this study (DHII, tBHQ, and menadione) protect against subsequent menadione exposure in Hepa-1 cells; this protection is correlated with the induction of mRNAs and enzymes of the [*Ah*] gene battery. The DHII-mediated induction process requires a functional AHR and acts via the AhRE, whereas the tBHQ- and menadione-mediated induction processes do not require a functional AHR, but rather operate via the EpRE (Fig. 5). Based upon these results, we anticipate that the putative EpRE binding sites found in the 5′ regulatory region of the murine [*Ah*] battery genes will be functional.

Acknowledgements—Supported by NIH grants P30-ES06096 and RO1-AG09235 and the Cancer Research Campaign, U.K.

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