Isoform-specific Induction of a Human Aldo-Keto Reductase by Polycyclic Aromatic Hydrocarbons (PAHs), Electrophiles, and Oxidative Stress: Implications for the Alternative Pathway of PAH Activation Catalyzed by Human Dihydrodiol Dehydrogenase

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ABSTRACT

Human dihydrodiol dehydrogenase (DD) isoforms are aldol-keto reductases (AKRs) that activate polycyclic aromatic hydrocarbons (PAHs) by oxidizing trans-dihydrodiol proximate carcinogens to reactive and redox-active ortho-quinones. Of these, human AKR1C1 (DD1) and AKR1C2 (DD2) oxidize trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene to the cytotoxic and genotoxic metabolite benzo[a]pyrene-7,8-dione (BPQ) with the highest catalytic efficiency. Exposure of HepG2 cells to a panel of inducers revealed that mRNA encoding one or more human AKR1C member(s) was induced (3- to 10-fold) by benzo[a]pyrene and other polycyclic aromatic compounds (bi-functional inducers), electrophilic Michael acceptors and phenolic antioxidants (monofunctional inducers), and reactive oxygen species (ROS). The induction of AKR1C mRNA by bi-functional inducers was delayed with respect to the induction of CYP1A1 mRNA, and AKR1C mRNA was not induced by the nonmetabolizable aryl hydrocarbon receptor ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). These data suggest that, in contrast to the CYPs, induction of AKR1C member(s) by PAHs and other bi-functional inducers is mediated indirectly via an antioxidant response element rather than a xenobiotic response element. Immunoblot and enzymatic assays confirmed that the increases in AKR1C mRNA were faithfully translated into functional AKR protein(s). The increased DD activity in HepG2 lysates was induced only by high concentrations of ursodeoxycholate, which suggested that AKR1C2 (DD2, bile-acid-binding protein) was not the isoform induced. RNase protection assays identified AKR1C1 (DD1) mRNA as the transcript which was up-regulated by mono- and bi-functional inducers and ROS in both human hepatoma (HepG2) and colon carcinoma (HT29) cells. BPQ, the electrophilic and redox-cycling product of the AKR1C1 reaction, also induced AKR1C1 expression. Thus, BPQ formation by AKR1C1 results in both a chemical (redox-cycling) and a genetic (AKR1C1 induction) amplification of ROS in PAH-exposed cells. Because ROS have been implicated in both tumor initiation and tumor promotion, the amplification of ROS by this pathway may play a significant role in PAH carcinogenesis.

INTRODUCTION

The AKRs are a growing superfamily of monomeric cytosolic NAD(P)H-dependent oxidoreductases (Mr ~34,000) that catalyze the interconversion of aldehydes and ketones to alcohols on drugs and xenobiotics. Their ability to generate alcohols for conjugation reactions on a variety of endogenous (steroids and prostaglandins) and exogenous substrates (aromatic aldehydes and ketones; Refs. 2–6) suggest that they play a central role in the metabolism of xenobiotics in a manner similar to the microsome-delimited CYPs.

Several AKR superfamily members are involved in carcinogen metabolism. The rat ethoxyquin-inducible aflatoxin aldehyde reductase (AKR7A1) catalyzes the reduction of aflatoxin dialdehyde to its corresponding diol and represents an important detoxification route for aflatoxin (7). In contrast, rat 3α-HSD/DD (AKR1C9) catalyzes the oxidation of PAH trans-dihydriodiol (proximate carcinogens) to reactive PAH o-quinones (8, 9) with the concomitant production of deleterious ROS (Ref. 10; Fig. 1). The propensity of PAH o-quinones to enter into futile redox cycles and amplify ROS and o-semiquinone radicals is in part responsible for the cytotoxic and genotoxic properties of the parent quinones (11–13). Such reactive and redox-active o-quinone metabolites may contribute to the complete carcinogenic potential of PAH. The diversion of PAH trans-dihydriodiol from diol-epoxides to o-quinones may thus provide an alternative pathway of PAH activation.

Multiple human DD isoforms (AKR1C1-AKR1C4) were recently cloned from HepG2 cells and the recombinant proteins were expressed in Escherichia coli and characterized with respect to their native proteins in human liver (14). All of the four human AKR1C subfamily members were capable of oxidizing the potent proximate carcinogen B[a]P-diol to BPQ in the following rank order: AKR1C2 > AKR1C1 > AKR1C4 > AKR1C3 (Table 1). These studies verified that multiple human homologues of rat 3α-HSD/DD are also capable of catalyzing this alternative pathway of PAH activation in human cells.

Genes that encode enzymes involved in PAH metabolism are often inducible by one or more classes of xenobiotics. One class of xenobiotics, monofunctional inducers (e.g., t-BHQ, EA), are electrophilic compounds that signal via an unclear mechanism to activate the expression of genes (GST Ya, NQO1, γGCS) carrying cis-elements termed EpRE/ARE(s) (for a review, see Ref. 15). Although it is likely that the ability of chemicals to signal via the EpRE/ARE is related to their electrophilicity (16), the actual signal transduction pathway may be mediated by an indirect effect such as alteration in intracellular redox status and/or oxidative stress (17). Polycyclic aromatic compounds (B[a]P, 3-MC, and β-NF), on the other hand, are classified as bifunctional inducers (16) which can induce gene expression via two distinct mechanisms: (a) unmetabolized Pahs bind directly to...
the AhR, which then activates gene expression from a cis-element termed the XRE. By this mechanism, PAHs induce XRE-containing genes such as the CYPs (18); and (b) PAHs are metabolized to electrophilic intermediates that then signal to EpRE/AREs located in the regulatory regions of EpRE/ARE-regulated genes. Thus, bifunctional inducers can induce both XRE-containing genes (CYP) and EpRE/ARE-containing genes (NQO1, GST, GCS) through two distinct mechanisms.

Recently, total human DD mRNA levels were shown to be highly overexpressed in both EA-resistant and EA-treated HT29 colon cells (19, 20), and subsequent studies demonstrated that this effect was due to increased RNA transcription as measured by nuclear run-on assays. These studies laid the precedent that one or more human AKR superfamily member(s) was inducible by monofunctional inducers. However, the high sequence identity that exists between the human AKR1C isoforms (80–98% nucleotide identity) precluded the exact identification of the individual transcript(s) up-regulated by EA exposure.

These studies sought to determine which, if any, of the four human AKR1C subfamily members that are involved in PAH metabolism were inducible by bifunctional inducers (PAH), monofunctional inducers (electrophilic compounds), and/or oxidative stress. In human hepatoma HepG2 cells, all of the three classes of inducers dramatically increased the expression of an AKR1C subfamily member at the level of mRNA, protein, and enzymatic activity. In contrast to the CYPs, evidence suggests that the induction of human AKR1C by PAH is via an EpRE/ARE rather than an XRE. RNase protection assays demonstrated that AKR1C1, a major human AKR1C isoform involved in the oxidation of B[a]P-diol to BPQ, is both the constitutive and inducible form of human AKR1C in both HepG2 hepatoma and HT29 colon carcinoma cells. Finally, BPQ, the electrophilic and redox-active B[a]P metabolite generated by AKR1C1, also induced AKR1C1 expression, thereby setting up a feed-back induction of the AKR1C1 gene. The implications for PAH activation are discussed.

MATERIALS AND METHODS

Chemicals and Reagents. Cell culture media and reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). Homogeneous recombinant AKR1C1 and AKR1C2 proteins were purified as described previously (14). The Hybspeed RNase Protection Assay reagents were purchased from Ambion (Austin, TX). The RNA transcription kit was from Stratagene (La Jolla, CA). Goat antirabbit IgG-horseradish peroxidase conjugate was from Bio-Rad ( Hercules, CA). Enhanced chemiluminescence (ECL) Western blotting reagent was purchased from Amersham (Arlington Heights, IL). b-NAD nucleotide cofactor was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). 1-Acenaphthenol, B[a]P, b-NF, DMNQ, EA, meso-hexestrol, t-BHQ, and ursodeoxycholic acid were purchased from Aldrich Chemical Co., (Milwaukee, WI). BPQ was synthesized according to published procedures (21). 3-MC and TCDD were obtained from the Midwest Research Institute (Kansas City, MO). All of the other chemicals used were of the highest grade available. Caution: All PAHs are potentially hazardous and should be handled in accordance with “NIH Guidelines for the Laboratory Use of Chemical Carcinogens.”

Table 1 Nomenclature of DD isoforms and their rates of BP-diol oxidation

<table>
<thead>
<tr>
<th>AKR</th>
<th>DD</th>
<th>Nucleotide identity</th>
<th>HSD</th>
<th>Common names</th>
<th>B[a]P-diol oxidized (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR1C1</td>
<td>DD1</td>
<td>100</td>
<td>20α-HSD</td>
<td>Bile-acid-binding protein</td>
<td>4.0</td>
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<tr>
<td>AKR1C2</td>
<td>DD2</td>
<td>98</td>
<td>Type 3 3α-HSD</td>
<td></td>
<td>5.4</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>DDX</td>
<td>87</td>
<td>Type 2 3α-HSD</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>AKR1C4</td>
<td>DD4</td>
<td>83</td>
<td>Type 1 3α-HSD</td>
<td>Chlordecone reductase</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Values taken from Burczynski et al. (14).

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Cell Culture. HepG2 hepatoma cells (passages 10–30) were maintained in Eagle’s MEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 and were passaged every 4 days at 1:10 dilution. For induction studies, 48 h before treatment 3 × 106 cells were seeded into 100-mm dishes containing fresh media. Two days later (~50–60% confluence), cells were exposed to various inducing agents. Aliquots (10 µl) of 1000 × stock solutions in DMSO were added to 10 ml of fresh culture medium, and cells were incubated for the indicated times before harvesting.

RNA Isolation and Northern Analysis. Cellular RNA was isolated using the Trizol reagent. Total RNA (10 µg) was separated by electrophoresis on 1.0% agarose/formaldehyde gels and transferred overnight to Duralon-UV membranes (Stratagene). Membranes were prehybridized in hybridization buffer (50% formamide, 10% dextran sulfate, 1 mM NaCl, and 1% SDS) with 100 µg/ml sheared salmon sperm DNA at 42°C for 2 h. After prehybridization, membranes were hybridized to 10³ dpm of [32P]dATP probes corresponding to (a) an 855-bp EcoRI fragment of the human colon DD1 cDNA (pBluescript-hcDD kindly provided by Dr. Paul Ciaccio and Dr. Ken Tew, Fox-Chase Cancer Center, Philadelphia, PA); (b) a 1-kb EcoRI fragment of the human CYP450 1A1 3′ UTR (phPl1–450–3′, ATCC # M25729); or (c) the 1kb reverse transcription-PCR amplified coding region of aldehyde reductase (AKR1A1 or DD3) that were labeled by random priming to a specific activity of greater than 10⁶ cpm/µg DNA. Hybridization was performed at 42°C for 16–24 h. After hybridization, blots were subjected to two high stringency washes with 0.1 × SSC plus 1% SDS at 60°C for 45 min and 30 min, respectively. Signal intensities were measured using the PhosphorImager system (Molecular Dynamics), and bands were exposed to X-ray film at −80°C overnight. For purposes of normalization, blots were stripped and reprobed with a 780-bp Psrl/Xbal fragment of human GAPDH labeled by random priming as above.

Lysate Preparation for Western Blotting and Enzymatic Assay of DD in Mammalian Cells. Cell lysates were obtained as described previously for rat hepatoma H4IIE cells (22). Briefly, 100-mm plates of HepG2 cells were washed twice with ice-cold 1× PBS and scraped into 10 ml of ice-cold 1× PBS. Cells were pelleted at 3000 rpm for 5 min at 4°C, decanted, and subjected to hypotonic and mechanical lysis by resuspension in 1 ml of ice-cold water followed by 20 strokes with a Dunce ground glass homogenizer on ice. Lysates were prepared by centrifugation at 14,000 × g for 10 min at 4°C, and then aliquots of the supernatants were subjected to Western blot analysis or enzymatic assay.

Western Blot Analysis. Portions of mammalian cell lysates (10 µg) or purified proteins (1.5 µg) were boiled for 5 min, cooled on ice, separated by SDS-PAGE and electro-transferred to nitrocellulose filters. Filters were incubated with polyclonal rabbit antirat 3O-HSD antiserum (antisera 71535) at a 1:1000 dilution (18). Immunoblots were developed by incubation with goat antirabbit IgG horseradish peroxidase conjugate using enhanced chemiluminescence detection. No bands were observed with preimmune serum.

Enzymatic Assays of 1-Aacenaphthenol Oxidation. Enzyme assays were performed on cell lysates in 1.0 ml systems containing: 1 mM 1-acenaphthenol and 1 mM NAD+ in 50 mM Tris-Cl buffer (pH 7.5) at 25°C. Initial velocities were determined on a Beckman DU640 spectrophotometer by measuring the change in absorbance of pyridine nucleotide at 340 nm (ε = 6200 M⁻¹ cm⁻¹).

RNase Protection Assay. Linearized plasmid templates for generating sense transcripts of the four human AKR1C cDNAs were prepared from pBluescript-hcDD (AKR1C1) or by inserting reverse transcription-PCR amplified coding sequences for AKR1C2, AKR1C3, and AKR1C4 (14) into pCRII (Invitrogen). In vitro transcription was performed using the RNA transcription kit and the appropriate T3, T7, or SP6 polymerases. The AKR1C1 and AKR1C2 antisense riboprobes used in these studies were generated by linearizing pBluescript-hcDD (T3 orientation) or pCRII-AKR1C2 (T7 orientation) with NcoI or MstI, respectively, followed by synthesis with the appropriate polymerase in the presence of [32P]UTP to generate 500– and 480-bp riboprobes, respectively. Labeled riboprobes were precipitated with 30 µg RNA, denatured by boiling, hybridized at 65°C for 20 min, and incubated with RNase A:T1 for 30 min. Protected fragments using the AKR1C1 riboprobe (AKR1C1, 500 bp; AKR1C2, 332 bp; AKR1C3, 250 bp; and AKR1C4, 178 bp) or the AKR1C2 riboprobe (AKR1C2, 488 bp; AKR1C1, 400 bp; AKR1C3, 250 bp; and AKR1C4, 178 bp) were analyzed by electrophoresis on a 4.5% acrylamide/8 M urea gel.

RESULTS

Comparison of the Profiles of Human AKR and CYP1A1 mRNA Induction by Xenobiotics in HepG2 Cells. To determine whether human AKR1C subfamily members (AKR1C1–AKR1C4) were responsive to bifunctional (B[β]P, 3-MC, β-NF) or monofunctional (EA, t-BHQ) inducers or oxidative stress (DMNQ, H2O2), HepG2 cells were exposed for 12 h to the various treatments and AKR1C mRNA levels were examined by Northern blot analysis (Fig. 2, A and B). Blots were stripped and reprobed to examine the expression of CYP1A1 mRNA, which is regulated only by an XRE. It was found that one or more human AKR1C members were constitutively expressed in untreated HepG2 cells, as opposed to CYP1A1 mRNA, which was undetectable (Fig. 2A). Furthermore, human AKR1C member(s) were induced by all three classes of inducers to levels approximately 3- to 10-fold higher than controls. In contrast, CYP1A1 was solely responsive to bifunctional inducers. The lack of response of CYP1A1 to monofunctional inducers is consistent with the previous finding that this gene is regulated by bifunctional inducers solely via the AhR-dependent XRE signaling pathway (18). Because the AKR1C1 probe used in this study recognizes at least four different in vitro synthesized AKR1C isoform transcripts (data not shown), these data suggest that the induction of at least one human AKR1C member by monofunctional inducers is mediated by an EpRE/ARE. However, they do not rule out the possibility that one or more human AKR1C members are also regulated by bifunctional inducers via an XRE-dependent mechanism as well. Another less closely related human AKR superfamily member that can be distinguished by Northern blot analysis is aldehyde reductase (AKR1A1). AKR1A1 was constitutively expressed in HepG2 cells but was not responsive to any of the inducers tested (Fig. 2A).

Human AKR1C subfamily member(s) were also found to be induced by ROS. In contrast, neither CYP1A1 nor AKR1A1 mRNA levels increased after ROS exposure. Treatment of HepG2 cells with H2O2 resulted in an ~8-fold increase in AKR1C RNA levels after 12 h (Fig. 2A, Lane 8). The compound DMNQ, a redox-active quinone that cannot be conjugated with GSH and is believed to exert its cellular effects via the intracellular generation of ROS, was also a potent inducer of AKR1C mRNA levels (~9-fold, Fig. 2A, Lane 7).

Time Course of AKR1C mRNA Induction by the Bifunctional Inducer β-NF and Lack of Induction by the AhR Ligand TCDD. To determine whether the induction of any human AKR1C member(s) by bifunctional inducers was consistent with an XRE-dependent or an EpRE/ARE-dependent mechanism, we compared the kinetics of AKR1C and CYP1A1 mRNA induction in HepG2 cells after exposure to the bifunctional inducer β-NF (Fig. 3A). CYP1A1 mRNA was induced by β-NF after only 1 h, indicating that XRE-dependent CYP1A1 gene expression in HepG2 cells is stimulated by ligands within a very short time course. AKR1C mRNA levels, on the other hand, did not increase until more than 4 h after β-NF exposure, which is consistent with a requirement for the metabolism of β-NF to an electrophilic species (16) before AKR1C expression is induced. These data suggest that human AKR1C expression is up-regulated by an EpRE/ARE-type mechanism, and that functional XREs are absent from the regulatory regions of human AKR1C genes.

To confirm this hypothesis, the ability of the nonmetabolizable AhR ligand TCDD to induce AKR1C mRNA was also tested (Fig. 3B). Although TCDD caused a robust increase in CYP1A1 mRNA levels in a time-dependent fashion, it failed to induce AKR1C mRNA even after 6 h. The lack of responsiveness of AKR1C mRNA to
was harvested, and 50 μM final concentration. Total RNA was harvested from cells at the indicated time points, electrophoresed, transferred to membranes, and then probed sequentially with probes corresponding to the AKR1C subfamily and CYP1A1 as described previously in “Materials and Methods.” Arrows, time points at which a 2-fold induction was assessed.

**Effects of AKR1C Inhibitors on 1-Aacenaphthenol Oxidation Catalyzed by Induced HepG2 Cell Lysates.** To verify that the increases in 1-acenaphthenol oxidation measured in HepG2 cell lysates were catalyzed by increased levels of AKR1C protein, the ability of the nonisoform selective AKR1C inhibitor meso-hexestrol (2) to inhibit this activity was assessed. Meso-hexestrol caused a dose-dependent inhibition of 1-acenaphthenol oxidation induced by EA, t-BHQ, and β-NF in HepG2 lysates yielding the same IC₅₀ values in all three cases (Fig. 5A), which indicated that all three xenobiotics likely induce the same AKR1C isoform in HepG2 cells.

To begin to address which AKR1C isoform was responsible for the increased enzymatic activity in HepG2 cells, we examined the effects of the AKR1C-selective inhibitor ursodeoxycholic acid. AKR1C2 (DD2) is also known as bile-acid-binding protein and is approximately 1000-fold more sensitive to inhibition by bile acids than other AKR1C members (4). A comparison of the IC₅₀ values for the inhibition of 1-acenaphthenol oxidation catalyzed by homogenous recombinant AKR1C1, homogenous recombinant AKR1C2, and EA-induced HepG2 cell lysates demonstrated that the increased activity in HepG2 cells was not catalyzed by AKR1C2 because ursodeoxycholate was a weak inhibitor of the measured activity (Fig. 5B).

TCDD confirms that the likely mechanism of AKR1C induction by parent PAH involves their metabolism to electrophilic species that then signal via an EpRE/ARE-dependent pathway.

**Induction of Human AKR1C Protein and Enzymic Activity.** To determine whether the observed increases in AKR1C mRNA expression were translated into increased levels of functional protein, HepG2 cells were exposed to β-NF, t-BHQ, or EA for 72 h, and cytosols were harvested at the indicated time points for immunoblotting and enzymatic assay. The four recombinant human AKR1C members are weakly immunoreactive with the polyclonal rabbit-antibody and enzymatic assay. The four recombinant human AKR1C members are weakly immunoreactive with the polyclonal rabbit-antibody and homogenous recombinant AKR1C1, homogenous recombinant AKR1C2, and EA-induced HepG2 cell lysates demonstrated that the increased activity in HepG2 cells was not catalyzed by AKR1C2 because ursodeoxycholate was a weak inhibitor of the measured activity (Fig. 5B).

To demonstrate that the increase in AKR1C protein also reflects an increase in catalytic activity, aliquots of HepG2 cell lysates were assayed for their ability to oxidize 1-acenaphthenol. This substrate is turned over with a high kₐ₄₅ by all of the four human AKR1C isoforms, and uninduced HepG2 lysates possess measurable basal rates of 1-acenaphthenol oxidation. The time-dependent increases in specific activity for 1-acenaphthenol oxidation observed in HepG2 cell lysates after treatment with β-NF, EA, and t-BHQ (Fig. 4B) demonstrate that the increases in AKR1C mRNA and protein are accompanied by similar increases in catalytic activity.

Fig. 3. Lack of involvement of XRE in human AKR1C induction by PAH. A, kinetics of mRNA induction by the bifunctional inducer β-NF. HepG2 cells (3 × 10⁶) were seeded into 100-mm dishes and 2 days later, stock solutions of β-NF in DMSO were added to fresh media to yield 50 μM final concentration. Total RNA was harvested from cells at the indicated time points, electrophoresed, transferred to membranes, and then probed sequentially with probes corresponding to the AKR1C subfamily and CYP1A1 as described previously in “Materials and Methods.” Arrows, time points at which a 2-fold induction appears. B, the nonmetabolizable AhR ligand TCDD fails to induce AKR1C expression but potently stimulates CYP1A1. HepG2 RNA was harvested from cells treated with 100 nM TCDD for the indicated time points and analyzed for AKR1C and CYP1A1 expression.
To distinguish between these possibilities and to confirm the results of the first RNase protection assay, a second antisense riboprobe directed against the AKR1C2 coding sequence was used. Because this riboprobe encompassed only a portion of the coding sequence, the protected fragments for the in vitro synthesized transcripts and transcripts present in HepG2 cells should be of identical size. The AKR1C2 antisense riboprobe yielded a 488-bp protected fragment for AKR1C2, a 400-bp protected fragment for AKR1C1, a 250-bp fragment for AKR1C3, and a 178-bp fragment for AKR1C4. This RNase protection assay clearly and unambiguously demonstrated that the transcript encoding AKR1C1 was constitutively expressed in resting HepG2 cells, and that this isoform was also strongly induced after exposure to EA, β-NF, and H2O2 (Fig. 6B). A similar result was observed in HT29 colon carcinoma cells (Fig. 6C), indicating that this isoform-specific induction of AKR1C1 is conserved in both human liver- and human colon-carcinoma cell lines.

Identification of AKR1C1 as the Inducible AKR1C Isoform by RNase Protection Assay. To determine the exact identity of the inducible AKR1C transcript(s) in HepG2 cells, RNase protection assays were performed. In an initial set of experiments, an antisense riboprobe spanning the 3’ end of the pBluescript-hcDD clone (AKR1C1) was used that unequivocally demonstrated that AKR1C1 (and to a weaker extent, AKR1C3) was significantly induced by all three classes of inducers in HepG2 cells (Fig. 6A). However, this riboprobe contained a portion of the 3’UTR of AKR1C1, whereas AKR transcripts synthesized in vitro from the other cloned AKR1C cDNAs lacked this region. Although the results of this initial RNase protection assay demonstrated the robust induction of AKR1C1, it also gave several closely related protected fragments that could arise from: (a) AKR1C1 3’UTR heterogeneity; (b) incomplete protection/secondary structure of the AKR1C1 transcript; or (c) the existence of significant homology between the 3’UTRs of AKR1C1 and AKR1C2 resulting in protected fragments for full length AKR1C2 transcripts from HepG2 cells (Fig. 6A, Lanes 5–12) which are larger than those observed when only the control AKR1C2 coding region is protected (Fig. 6A, Lane 2).

Fig. 4. Induction of functional human AKR1C enzyme by xenobiotics. A. immunoblot analysis of HepG2 lysates after exposure to xenobiotics. HepG2 cells were treated as described previously with the following concentrations of compounds: 50 μM β-NF, 50 μM EA, and 70 μM t-BHQ for 1–3 days and then lysates were prepared, analyzed by SDS-PAGE, and subjected to Western blot analysis as described in “Materials and Methods.” B. induction of 1-acenaphthenol oxidation by HepG2 lysates after exposure to xenobiotics. At the appropriate time points aliquots of HepG2 lysates were assayed for 1-acenaphthenol oxidation as in “Materials and Methods” and then analyzed for protein content using the Bradford detection reagent (Bio-Rad) to generate specific activities for each treatment. Data are expressed as fold induction of AKR1C relative to the DMSO solvent control. The mean ± S.E. for n = 3–9 is shown.

Identification of AKR1C1 as the Inducible AKR1C Isoform by RNase Protection Assay. To determine the exact identity of the inducible AKR1C transcript(s) in HepG2 cells, RNase protection assays were performed. In an initial set of experiments, an antisense riboprobe spanning the 3’ end of the pBluescript-hcDD clone (AKR1C1) was used that unequivocally demonstrated that AKR1C1 (and to a weaker extent, AKR1C3) was significantly induced by all three classes of inducers in HepG2 cells (Fig. 6A). However, this riboprobe contained a portion of the 3’UTR of AKR1C1, whereas AKR transcripts synthesized in vitro from the other cloned AKR1C cDNAs lacked this region. Although the results of this initial RNase protection assay demonstrated the robust induction of AKR1C1, it also gave several closely related protected fragments that could arise from: (a) AKR1C1 3’UTR heterogeneity; (b) incomplete protection/secondary structure of the AKR1C1 transcript; or (c) the existence of significant homology between the 3’UTRs of AKR1C1 and AKR1C2 resulting in protected fragments for full length AKR1C2 transcripts from HepG2 cells (Fig. 6A, Lanes 5–12) which are larger than those observed when only the control AKR1C2 coding region is protected (Fig. 6A, Lane 2).

Fig. 5. Inhibition of 1-acenaphthenol oxidation in HepG2 lysates with the AKR1C inhibitors meso-hexestrol (A) and ursodeoxycholic acid (B). A. 50 μg of induced HepG2 lysates were assayed for 1-acenaphthenol oxidation in the presence of increasing concentrations of meso-hexestrol, and the percent inhibition at each concentration of inhibitor were then fitted to sigmoidal curves in Sigma Plot version 4.0 to generate IC50 values for each of the treatments. B. purified AKR1C1 (DD1) and AKR1C2 (DD2) enzymes (2 μg), or 50 μg of induced HepG2 lysate were assayed for 1-acenaphthenol oxidation in the presence of increasing concentrations of the bile acid ursodeoxycholic acid, and fitted to yield IC50 values for each experiment.
more stable than originally anticipated. Thus, BPQ, a product of
AKR1C1, is capable of inducing the expression of an enzyme
responsible for its own formation, which results in continued activation of
trans-dihydrodiols via the DD pathway after PAH exposure.

DISCUSSION

We sought to determine the identity of human AKR1C subfamily
member(s) that are regulated by classes of inducers known to induce
the expression of xenobiotic metabolizing enzymes. Our studies dem-
monstrate that the xenobiotic-inducible transcript in human hepatoma
and colon carcinoma cells is AKR1C1. AKR1C1 represents the first
human member of the AKR superfamily to be identified as responsive
to mono- and bifunctional inducers and oxidative stress. It joins a
growing list of AKR superfamily members from other species that are
also inducible by similar xenobiotic insults, including the ethoxyquin-
inducible aflatoxin aldehyde reductase (AKR7A1) from rat liver (8)
and the H2O2-inducible aldose reductase from rat vascular smooth
muscle (23). These similar profiles of genetic regulation across species
likely indicate an important evolutionarily conserved role for
members of the AKR superfamily in the cellular response to xenobi-
totic exposure.

The delayed kinetics of AKR1C1 induction by β-NF and the lack
of AKR1C1 induction in response to TCDD exclude a role for an
XRE in the induction mechanism. Instead, the data support a role for
an EpRE/ARE-like mechanism in the induction of AKR1C1 by bi-
functional inducers. In these studies, the identification of the AKR1C1
transcript as the inducible AKR1C isoform in human cells should
allow the rapid location of the putative EpRE/ARE in the regulatory
regions of the human AKR1C1 gene. To date, no functional EpRE/
ARE has been detected within the human AKR1C1 gene promoter
(24). There are six AP-1 like sites in the proximal 819 bp of the 5′
flanking region, however none of these sites possess 100% sequence
identity with the currently accepted core consensus sequence for the
ARE, TGACNNNGC (25).

It is interesting to note that the fold induction of AKR1C1 by
DMNQ and EA is similar. DMNQ is a redox-active quinone but is not a Michael acceptor (a compound that readily reacts with nucleophiles because of the presence of an \(\alpha, \beta\)-unsaturated ketone), whereas EA is a Michael acceptor that is not redox-active. Nonetheless, both compounds induce AKR1C1 expression to a similar extent. The data with DMNQ rule out a requirement for the inducer to undergo Michael addition chemistry before it can signal to the EpRE/ARE. However, a common eventual cellular outcome of exposure to DMNQ, EA, and ROS is depletion of reduced GSH levels which could then lead to a pro-oxidant environment, and this may be an important determinant in the induction of phase II enzyme gene expression by both ROS and Michael acceptor xenobiotics.

Human AKR1C1 and human CYP1A1 compete for B[a]P-diol and turn over the proximate carcinogen to BPQ or anti-BPDE, respectively, with similar turnover rates (0.1 and 0.5 min\(^{-1}\), respectively; Refs. 14 and 26). AKR1C1 is constitutively expressed in a liver-derived cell line whereas CYP1A1 is not. This implies that once CYPs have been induced by B[a]P and catalyze its conversion to B[a]P-diol, any B[a]P-diol entering the cytosolic compartment will be available as substrate for resting, uninduced levels of AKR1C1. The selective induction of AKR1C1 by oxidative stress or electrophilic compounds in responsive cell types would result in a further increase in the conversion of B[a]P-diol to BPQ, in some instances by up to 10-fold. BPQ is a moderately cytotoxic agent: exposure of HepG2 cells to BPQ reduces cell viability by depleting GSH (11). Additionally, BPQ is a potent genotoxin; it has the potential to form both stable and depurinating adducts with DNA (27, 28); and it acts as a potent chemical nuclease \textit{in vitro} and in primary cell culture (12). Selective induction of AKR1C1 (relative to CYP1A1) suggests that under these circumstances the spectrum of DNA damage associated with PAH \(\alpha\)-quinone generation will increase, including the formation of oxidatively damaged bases. Indeed, increased levels of 8-'OH-dG have been observed in DNA from rodents or human mammary epithelial cells exposed to parent B[a]P without a satisfactory explanation to date (29, 30). Furthermore, the incidence of tumors in DMBA-treated mice is significantly increased in mice which are metallothionein deficient, implying that free radicals and/or oxidative stress plays a significant role in the murine model of DMBA-induced skin carcinogenesis (31). Recently we have shown that the \textit{trans}-dihydrodiol of DMBA is an excellent substrate for all four human AKR1C members, including the inducible isoform AKR1C1 (32).

In preliminary disposition experiments we have shown that BPQ formation is enhanced in EA-induced HepG2 cells after administration of B[a]P-diol. In addition, overexpression of AKR1C9 in a stably transfected MCF7 cell line enhances the cytotoxicity of administered B[a]P-diol and this effect is blocked by DD inhibitors (33). These studies verify the metabolic and biological significance of inducing AKR1C isoforms in the presence of PAH trans-dihydrodiol proximate carcinogens.

Our studies demonstrated that ROS can potently induce AKR1C1 expression. The synthetic redox-cycling antitumor quinone Adriamycin has also been shown to induce \textit{GST Ya} gene expression in rat H4II cells (17). Because AKR1C1 converts B[a]P-diol to an endogenously generated redox-cycling quinone BPQ, we determined whether the BPQ produced by this pathway could feedback and increase AKR1C1 gene expression still further. Indeed, low micromolar concentrations of BPQ consistently stimulated AKR1C1 mRNA levels by ~3-fold. Although physiological enzymes employ feedback inhibition when product levels reach a threshold, it is not incumbent upon enzymes involved in xenobiotic metabolism to be regulated in a similarly altruistic manner. Thus BPQ formation by AKR1C1 will result in both a chemical (redox-cycling) and genetic (AKR1C1-induction) amplification of ROS production in PAH-exposed cells as depicted in Fig. 8. The consequences of ROS production result in a variety of cellular events in a dose-dependent manner. Modest levels of ROS can lead to the formation of oxidatively damaged bases, e.g., 8-'OH-dG, which can give rise to G to T transversions (34). Such mutations are believed to

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to represent initiating events. Further generation of a prooxidant state leads to activation of protein kinase C and tumor promotion and results in an overall increase in genomic instability (35–37). Finally, higher levels of ROS can lead to cell death either by apoptosis and/or necrosis (38). Depending on the extent of the AKR1C1-catalyzed reaction in various human cell types, it is rational to suggest that ROS derived from this pathway may certainly contribute to the complete carcinogenic potential of PAH.

In summary, these studies identify the AKR1C1 isoform as an inducible AKR in human HepG2 hepatoma and HT29 colon carcinoma cells. AKR1C1 is inducible by multiple classes of xenobiotics including mono- and bifunctional inducers and ROS. Induction of AKR1C1 by parental BaP and other PAH appears to be mediated indirectly by an EpRE/ARE, rather than an XRE. The AKR gene superfamily represents yet another family of PAH metabolizing enzymes possessing individual isoforms that are coordinately induced along with other phase II (de)toxification enzymes in response to PAH and other xenobiotics. The feedback induction of AKR1C1 by both BPQ and ROS also suggests that under conditions of chronic oxidative stress, elevated levels of the ROS inducible AKR1C1 will enhance the turnover of PAH trans-dihydriodiol to redox-cycling o-quinones and further exacerbate cellular damage mediated by PAH via a ROS-dependent pathway.

REFERENCES


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Isoform-specific Induction of a Human Aldo-Keto Reductase by Polycyclic Aromatic Hydrocarbons (PAHs), Electrophiles, and Oxidative Stress: Implications for the Alternative Pathway of PAH Activation Catalyzed by Human Dihydropyridine Dehydrogenase

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