Genotoxic Polycyclic Aromatic Hydrocarbon ortho-Quinones Generated by 
Aldo-Keto Reductases Induce CYP1A1 via Nuclear Translocation of the 
Aryl Hydrocarbon Receptor

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ABSTRACT

Procarcinogenic polycyclic aromatic hydrocarbons (PAHs) induce their own metabolism and activation by binding to the cytosolic aryl hydrocarbon receptor (AhR), which then translocates to the nucleus and activates CYP1A1 gene transcription via xenobiotic response elements (XREs). Although the AhR demonstrates a strict specificity for planar aromatics, nonplanar (trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene) also induced CYP1A1 expression in HepG2 cells over a delayed timecourse (~6–12 h), suggesting a requirement for (trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene) metabolism. Aldo-keto reductase (AKR) inhibitors blocked this effect, suggesting that benzo(a)pyrene-7,8-dione (BPQ), a planar PAH o-quinone generated by AKRs, was the downstream inducer. BPQ was found to be a potent and rapid inducer of CYP1A1, with an EC50 value in HepG2 cells identical to that of the parent benzo(a)pyrene. BPQ was a more potent inducer of CYP1A1 when compared with the 1,6-, 3,6-, and 6,12-benzo(a)pyrene-diones. Multiple PAH o-quinones caused induction of CYP1A1, demonstrating that this was a general property of AKR-generated PAH o-quinones. HepG2-101L cells stably transfected with a XRE-luciferase construct showed that BPQ activated CYP1A1 transcription via a XRE-dependent mechanism. BPQ failed to induce CYP1A1 in AhR-deficient and AhR nuclear translocator-deficient murine hepatoma cell lines and confirmed that induction of CYP1A1 was AhR and AhR nuclear translocator-dependent. Electrophoretic mobility shift assays demonstrated the specific appearance of BPQ-activated AhR in the nucleus, and immunofluorescence studies confirmed that BPQ mediated nuclear translocation of the AhR. Classical bifunctional inducers elevate CYP1A1 expression via a XRE and are subsequently converted by CYP1A1 to electrophiles that induce phase II enzymes via an electrophilic response element/antioxidant response element. PAH o-quinones represent a novel class of bifunctional inducer because they are electrophiles produced by phase II enzymes that simultaneously induce phase I enzymes via a XRE and phase II enzymes via an electrophilic response element/antioxidant response element (see also M. E. Burczynski et al., Cancer Res., 59: 607–614, 1999). This study shows that the AhR provides the only known mechanism by which genotoxic PAH o-quinones generated in the cytosol can be targeted to the nucleus with specificity.

INTRODUCTION

PAHs (B(a)P, for example) are ubiquitous environmental procarci-

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The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; B(a)P, benzo(a)pyrene; AhR, aryl hydrocarbon receptor; AKR, aldo-keto reductase; ARE/EpRE, antioxidant response element/electrophilic response element; BPQ, benzo(a)pyrene-7,8-dione; CYP, cytochrome P450; B(a)P-diol, (trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; ARE, antioxidant response element; EpRE, electrophilic response element; XRE, xenobiotic response element; anti-BPDE, (anti-trans-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; TDCC, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ARNT, AhR nuclear translocator; ROS, reactive oxygen species; 6MPA, 6-mercaptopurine; proteinase; GAPDH, glyceralde-

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y-3-phosphate dehydrogenase; BAQ, benz[a]anthracene-3,4-dione; NQO, naphthalene-oxide effects (1–3). A major pathway of PAH metabolic activation proceeds through the CYP-mediated generation of PAH trans-dihydriodiol proximate carcinogens (4). Once formed, PAH trans-dihydriodiol can undergo further oxidative metabolism to reactive electrophiles via two distinct enzymatic pathways (Fig. 1).

In the first pathway, human CYPs catalyze a secondary epoxidation vicinal to the trans-dihydriodiol to yield bay region diol-epoxides (4, 5). These diol-epoxides are protected from hydrolysis by epoxide hydrolase due to steric hindrance in the bay region (6). Their resistance to hydrolysis may permit them to reach the nucleus from their site of formation, although the exact process whereby diol-epoxides access the nucleus is not known. PAH diol-epoxides are well-characterized metabolites that react readily with mutational hot spots in DNA to form stable adducts both in vitro and in vivo (7, 8). If unrepaird, anti-BPDE-DNA adducts result in misreplication and mutagenesis (9, 10).

A second pathway of PAH activation is catalyzed by AKRs (11, 12). The AKRs are monomeric cytosolic NADP(H)-dependent oxidoreductases (~34 kDa), and several human isoforms (AKR1C1–AKR1C4) catalyze the oxidation of PAH trans-dihydriodiol to PAH o-quinones (12–13). In the case of B(a)P, the proximate carcinogen B(a)P-diol is converted by AKRs to BPQ. PAH o-quinones are electrophilic metabolites that enter futile redox cycles and amplify ROS multiple times (15). PAH o-quinones are formed in rat hepatocytes by AKR1C9 and are both cytotoxic and genotoxic in vitro (16, 17). These quinones form stable and depurinating adducts with DNA, and their propensity to redox cycle could lead to oxidative damage of DNA (e.g., formation of 8′-hydroxy-2-deoxyguanosine, strand scission, and base propensities; Refs. (18–20). PAH o-quinones are generated by AKRs in the cytosolic compartment, and how PAH o-quinones gain access to the nucleus is presently unknown. In disposition studies in primary rat hepatocytes, a significant amount of 20 μM [3H]BPQ (30%) was sequestered into the cell pellet (DNA, RNA, and protein) within 0.5 h (17). Treatment of primary rat hepatocytes with these toxicological concentrations of BPQ results in extensive strand scission of the genomic DNA. These data suggest that BPQ generated in the cytosol, like anti-BPDE formed in the microsomes, can reach the nucleus.

PAHs are considered bifunctional inducers: they enhance the expression of both the CYPs and phase II (de)toxication enzymes (including the AKRs) through two distinct mechanisms (21). In the first mechanism, PAHs bind directly to a cytosolic receptor termed the AhR that demonstrates a strict specificity for planar aromatic compounds (22). Upon binding ligand, the cytosolic AhR dissociates from heat shock protein 90 and is rapidly translocated into the nucleus, where it forms a complex with its heterodimeric partner, ARNT. The ligand-bound AhR/ARNT complex then robustly activates the expres-

1,2-dione: DMBAQ, dimethylbenz[a]anthracene-3,4-dione; DD, dihydriodiol dehydrogenase

[trans-1,2-dihydriodiol-1,2-diol dehydrogenase (EC 1.3.1.20)]

The nomenclature for the AKR superfamily was proposed by Jez et al. (Ref. 14) and adopted at the 8th International Symposium on Enzymology and Molecular Biology of Carboxyl Metabolism in Deadwood, South Dakota, June 29–July 3, 1996.
sion of a battery of genes containing XREs within their regulatory regions, including CYP1A1 (for reviews, see Refs. 23 and 24; Fig. 2).

In the second mechanism, PAHs are activated by CYP1A1 metabolism to unidentified electrophiles. These electrophilic intermediates signal by an incompletely characterized mechanism to activate components (possibly activator protein 1 and/or the Nrf2/maf families of transcription factors) to bind and activate transcription from response elements termed AREs or EpREs present in the regulatory regions of responsive genes encoding phase II (de)toxification enzymes (Refs. 25–28, for a review, see Ref. 29). BPQ is formed in human hepatoma (HepG2) cells by AKR1C1 and autoregulates AKR1C1 expression, most likely via an ARE/EpRE-like mechanism (30).

The present studies sought to determine whether downstream electrophilic metabolites of PAH might also feedback stimulate the expression of the CYP1A1 gene. In HepG2 cells, B(a)P-diol robustly enhanced CYP1A1 mRNA levels with delayed kinetics, implying that a downstream metabolite of B(a)P-diol was the actual CYP inducer. Subsequent studies identified PAH o-quinones generated by human AKRs as the inducers. PAH o-quinones induce CYP1A1 via the AhR signaling pathway, demonstrating that these electrophilic metabolites represent novel bifunctional inducers that require no further metabolism to simultaneously induce both phase II (de)toxification enzymes (via an EpRE/ARE due to their electrophilic/redox-active nature) and phase I activating enzymes (via the XRE due to their restored planarity). These studies also imply that the cytosolic colocalization of AKR enzymes and the AhR provides a mechanism whereby PAH o-quinones generated in the cytosol can be targeted to the nucleus with specificity. The nuclear targeting of these redox-active o-quinones by the AhR may contribute to the spectrum of oxidative DNA damage observed after exposure of cells to PAH.

MATERIALS AND METHODS

**Chemicals and Reagents.** Cell culture media and reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). The Dual Luciferase Reporter Assay System was obtained from Promega Corp. (Madison, WI). Polyclonal rabbit anti-murine AhR antisera was purchased from Biomol (Plymouth Meeting, PA). B(a)P, benzo(a)anthracene, 7,12-dimethylbenzo(a)anthracene, 6MPA, and ursochoxycholic acid were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). NPQ and BPQ were synthesized according to previously published procedures (31, 32). BAQ and DMBAQ were gifts from Dr. Ronald G. Harvey (Ben May Institute, University of Chicago, Chicago, IL). TCDD, B(a)P-diol, B(a)P-1,6-dione, B(a)P-3,6-dione, and B(a)P-6,12-dione were obtained from the National Cancer Institute Midwest Research Institute (Kansas City, MO). All other chemicals used were of the highest grade available. All PAHs are potentially hazardous and should be handled in accordance with NIH Guidelines for the Laboratory Use of Chemical Carcinogens.

**Cell Culture.** HepG2 hepatoma cells (passages 5–20) were maintained in Eagle’s MEM supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin solution. Hepa1c1c7, hepa1c1c4, and hepa1c1c12 cells (kindly provided by Dr. Oliver Hankinson, University of
California Los Angeles, Los Angeles, CA) were maintained in a-MEM (without nucleosides) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, and 2.5 μg/ml fungizone solution (Irvin Scientific, Santa Anna, CA). HepG2-101L cells stably expressing a portion of the CYP1A1 gene promoter (-1612/+292) that bears three consensus XREs (a kind gift from Dr. Robert H. Tukey, University of California, San Diego, La Jolla, CA) linked to a luciferase reporter were maintained in DMEM (with low glucose) supplemented with 5% non-heat-inactivated FCS, 5% non-heat-inactivated NuSerum IV, and 0.4 mg/ml G418 to maintain selection. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 and passed every 5 days at a 1:2 dilution (HepG2, hepalc1c7, hepalc1c4, and hepalc1c12 cell lines) or every 7 days at 1:4 dilution (HepG2-101L). To measure either the induction of CYP1A1 RNA or the nuclear translocation of the AhR by electrophoretic mobility shift assays, 48 h before treatment, 3 × 10⁶ hepatoma cells were seeded into 10-cm dishes containing fresh media. Two days later (~60–70% confluence), cells were exposed to the various inducers. Aliquots (100 μl) of 100 X stock solutions in DMSO were added to 10 ml of fresh culture medium, and cells were incubated for the indicated times before harvesting. For XRE-luciferase reporter gene assays, 24 h before treatment, 1 × 10⁶ cells were seeded into 6-well plates. One day later (70–80% confluence), cells were treated with the various inducers 12 h 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, La Jolla, CA). Membranes were prehybridized in hybridization buffer (30% formamide, 10% dextran sulfate, 1 M NaCl, and 1% SDS) with 100 μg/ml sheared salmon sperm DNA at 42°C for 2 h. After prehybridization, membranes were hybridized to 10⁵ dpm of [α-32P]dATP probes corresponding to either: (a) a 1-kb EcoRI fragment of the human CYP450 1A1 3' untranslated region (pHP1-450-3', ATCC T57259); (b) a 1-kb EcoRI fragment of the murine CYP450 1A1 (pMP1-450-3', ATCC 63006); or (c) an 855-bp EcoRI fragment of human AKR1C1 (hDd/Dd1) cDNA (kindly provided by Dr. Paul Ciaccio and Dr. Ken Tew, Fox Chase Cancer Center, Philadelphia, PA) 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 X 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 blots were exposed to X-ray film at −80°C overnight. For purposes of normalization, blots were stripped and reprobed with a 780-bp PstI/XbaI fragment of human GAPDH labeled by random priming as described above.

Luciferase Reporter Assays. The luciferase assay was carried out using the Dual Luciferase Reporter Assay system from Promega. At the end of the treatment period, HepG2-101L cells were rinsed twice in ice-cold PBS and then incubated in 1 ml of 1× passive lysis buffer for 15 min on an orbital shaker at room temperature. Lysates were centrifuged for 30 s at 14,000 rpm. Pelleted cells were resuspended in hypotonic lysis buffer [10 mM HEPES (pH 7.9) containing 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride] and centrifuged at 3,000 rpm for 5 min. Pelleted cells were resuspended in hypotonic lysis buffer and disrupted with 10 strokes in a Dounce homogenizer (>90% lysis by trypan blue exclusion). Nuclei were pelleted at 14,000 rpm for 30 min and then resuspended in 0.5× packed nuclear volume of (low salt) nuclear extraction buffer containing high salt (0.8 M KCl), samples were mixed on a tiltable for 30 min at 4°C. Samples were centrifuged at 14,000 × g for 30 s, and the supernatants (nuclear extracts) were assayed immediately for protein content using the Bradford reagent (BioRad) and used in subsequent electrophoretic mobility shift assays.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were performed using a double-stranded oligonucleotide containing a consensus XRE-binding site from the CYP1A1 promoter corresponding to −998 to −969 (5'-dTTTCCTGGCCTTCTCAGCAACGCTGGGCA-3') (italic letters correspond to the core XRE sequence) of the human CYP1A1 gene (DRE-983, as described previously in Ref. 34). After annealing, the probes were labeled with [α-32P]dATP using the Klenow fragment (to fill extra TT dinucleotide overhangs at either end of the annealed double-stranded oligonucleotide) and then purified through QiAquick nucleotide removal columns (Qiagen, Valencia, CA). Nuclear extracts (5 μg of protein) were incubated in binding buffer (25 mM HEPES (pH 7.9) containing 100 mM KCl, 0.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) in the presence of 2.5 μg of BSA, 0.2 μg of poly(dI-dC), and 50 fmol of 32P-labeled XRE oligonucleotide (20,000 cpm) in a total volume of 15 μl at room temperature for 30 min. The reaction mixture was then subjected to electrophoresis through a 4.5% polyacrylamide gel containing 1% TBE and 1% glycerol. The gels were dried under a vacuum for autoradiography (overnight exposure) and subsequent PhosphorImager analysis. For competition experiments, nuclear extracts were incubated before the addition of labeled oligonucleotide for 10 min at room temperature with an excess of unlabelled oligonucleotides and then incubated in the binding reactions described.

Cell Growth and Fixation for Immunofluorescence Microscopy. Hepalc1c7 cells were detached by trypsinization and seeded into 60-mm culture dishes containing sterile glass coverslips previously coated in a solution of collagen (100 μg/ml) for 1 h. Cells were seeded at 30% confluence and allowed to attach overnight. The following day, coverslips were washed with PBS at room temperature and then incubated in 4% paraformaldehyde (pH 7.4) for 15 min. After three rinses in PBS, coverslips were incubated in 0.05% Triton in PBS for 10 min.

Immunofluorescence Staining and Microscopy. Coverslips were blocked in 2% goat IgG in 0.05% Triton in PBS for 30 min. After aspiration, coverslips were incubated in a 1:50 dilution of primary antibody solution (rabbit antiamurine AhR) at room temperature for 90 min. After three rinses in 1× PBS, coverslips were incubated in a 1:1000 dilution of secondary antibody solution (goat antirabbit IgG fluorescein conjugate) at room temperature for 90 min in the dark. Coverslips were washed three times with 1× PBS and then mounted onto glass slides in mounting media. Once dry, fluorescence was observed with a Nikon Diaphot fluorescent microscope.

RESULTS

Delayed Induction of Human CYP1A1 Expression by B(a)P-diol. Treatment of HepG2 cells with either 10 nM TCDD or 10 μM B(a)P (data not shown) led to a robust and rapid induction of CYP1A1 mRNA within 1–3 h. When HepG2 cells were treated with 10 μM B(a)P-diol, CYP1A1 mRNA was not induced until 6–12 h (Fig. 3). The delayed but robust induction of CYP1A1 via B(a)P-diol suggested that this metabolite either (a) induced CYP1A1 expression via

Fig. 3. Delayed induction of CYP1A1 by B(a)P-diol. HepG2 cells (3 × 10⁶) were seeded into 10-cm dishes, and 2 days later, stock solutions of TCDD or B(a)P-diol in DMSO were added to fresh media to yield final concentrations of 10 nM TCDD or 10 μM [B(a)P-diol] in 0.1% DMSO. After the appropriate time points, total RNA was harvested, and 10 μg of RNA per lane were electrophoresed, transferred to membranes, and analyzed for CYP1A1 expression as described in “Materials and Methods.” GAPDH reprobing (data not shown) revealed equal loading of all lanes.
appropriate final concentrations. After a 30-min preincubation with AKR inhibitors, 5 mM ursodeoxycholate or 6MPA were added to fresh media to yield the appropriate range of final concentrations in 0.1% DMSO. After 6 h, total RNA was harvested, and 10 μg of RNA per lane were electrophoresed, transferred to membranes, and sequentially analyzed for CYP1A1 and GAPDH mRNA as described in “Materials and Methods.” Results for each time point were determined using PhosphorImager analysis and are expressed as the fraction of the maximal CYP1A1:GAPDH ratio observed with 30 nM TCDD.

**Inhibition of B(a)P-diol-induced CYP1A1 Expression by AKR Inhibitors.** B(a)P-diol can be metabolized to either the diol-epoxide anti-BPDE by human CYPs or to the ortho-quinone BPQ by human AKRs. The recent discovery that AKR1C1 is constitutively expressed in resting HepG2 cells, whereas CYP1A1 is not detectable (30), suggested that AKR1C1-dependent oxidation of B(a)P-diol to BPQ may be the requisite metabolic event responsible for the induction of CYP1A1 mRNA by the proximate carcinogen. To test this hypothesis, HepG2 cells were coincubated with B(a)P-diol in the presence of two AKR1C1 inhibitors of varying potency (Fig. 4). Both 6MPA and ursodeoxycholic acid inhibited the B(a)P-diol-dependent induction of CYP1A1 mRNA in HepG2 cells with IC50 values that were higher but approach the IC50 values observed for the inhibition of purified recombinant AKR1C1 in vitro, respectively (Ref. 30; data not shown). These data suggest that the AKR1C1-dependent oxidation of B(a)P-diol to BPQ is the metabolic event responsible for the induction of CYP1A1 in B(a)P-diol-treated HepG2 cells.

**CYP1A1 Induction Potencies of Various B(a)P Metabolites in HepG2 Cells.** To directly demonstrate that BPQ was capable of inducing CYP1A1 mRNA in HepG2 cells, dose-response curves for B(a)P and its CYP- and AKR-derived metabolites (B(a)P-diol, anti-BPDE, and BPQ) were compared with the prototypical CYP1A1 inducer TCDD (Fig. 5). The ED50 for the TCDD-dependent induction of CYP1A1 mRNA in HepG2 cells was approximately 1 nM. B(a)P and BPQ were nearly equipotent in their ability to induce CYP1A1 mRNA (ED50 ~1 μM), and both were less efficacious than TCDD. Importantly both compounds produced a rapid (~1 h) and robust induction of CYP1A1 mRNA, suggesting that neither inducer required metabolism to an active species. The finding that B(a)P is approximately 1000-fold less potent than the nonmetabolizable inducer TCDD is consistent with previous reports comparing TCDD and PAH (35). anti-BPDE was incapable of inducing CYP1A1 levels, even at the highest doses tested (30 μM). Thus, BPQ is the only downstream B(a)P-diol metabolite that acts as a direct inducer of CYP1A1 mRNA in HepG2 cells.

**Comparison of CYP1A1 mRNA Induction in HepG2 Cells by PAH ortho-Quinones and Extended Quinones of B(a)P.** To determine whether induction of CYP1A1 expression is a hallmark of all PAH quinones, a series of PAH quinones were screened as potential inducers of CYP1A1 (Fig. 6). In the first experiment, three extended quinones of B(a)P derived from CYP-generated phenol metabolites were compared with the AKR-generated ortho-quinone BPQ for their ability to induce CYP1A1 in HepG2 cells. BPQ was a superior inducer of CYP1A1 mRNA when compared with the 1,6-, 3,6-, and 6,12-benzo(a)pyrene-diones. The abilities of various PAH o-quinones generated by AKRs to induce CYP1A1 mRNA were widely disparate (Fig. 6). The rank order of potency was BPQ > BAQ > NQO > DMBAQ. This rank order inversely follows their reactivity with cellular thiols, e.g., glutathione (data not shown), suggesting that increased electrophilicity of PAH o-quinones may limit their ability to induce CYP1A1 mRNA in HepG2 cells.

**Transcriptional Activation of XRE-dependent Reporter Gene Expression by BPQ.** To determine whether the observed increases in steady-state CYP1A1 mRNA levels by BPQ were due to increased transcription of the CYP1A1 gene via a XRE, the ability of BPQ to induce XRE-dependent reporter gene expression was studied. HepG2-101L cells, which carry a stably integrated XRE-containing portion of
the CYP1A1 promoter (−1612/+292) fused to the luciferase reporter gene (36), were exposed to either B(a)P (positive control) or BPQ. Both B(a)P and BPQ efficiently stimulated transcription (∼20- and 13-fold, respectively) from the XRE-reporter (Fig. 7), demonstrating that BPQ mediates transcription of CYP1A1 mRNA via XREs located in the 5’ flanking region of the CYP1A1 gene.

BPQ Fails to Induce CYP1A1 mRNA in AhR- or ARNT-deficient Cells. Because classical XRE-dependent transcription is activated by ligand binding to the AhR, we next tested whether the induction of CYP1A1 mRNA by BPQ was AhR dependent. The ability of BPQ to induce CYP1A1 mRNA in wild-type (1c1c7), AhR-deficient (1c1c12), and ARNT-deficient (1c1c4) murine hepatoma (hepa1) cells was therefore determined. Whereas BPQ was able to induce CYP1A1 in wild-type hepa1c1c7 cells (Fig. 8), it failed to induce CYP1A1 in either AhR- or ARNT-deficient hepatoma cells.

BPQ Causes Translocation of the AhR to the Nucleus. To demonstrate the ability of BPQ to translocate the cytosolic AhR into the nucleus of human cells, HepG2 cells exposed to BPQ or to the classical AhR ligands TCDD and B(a)P, and nuclear extracts were prepared. Incubation of equal amounts of nuclear extracts from untreated cells with 32P-labeled XRE from the CYP1A1 gene (−983 to −964 bp) failed to induce the formation of a specific AhR-XRE complex. However, nuclear extracts from TCDD- and BPQ-treated cells resulted in the appearance of a specific gel-shifted complex.

These results clearly indicate that BPQ, like TCDD and B(a)P, activates CYP1A1 expression via an AhR/ARNT-dependent pathway. BPQ Causes Translocation of the AhR to the Nucleus. To demonstrate the ability of BPQ to translocate the cytosolic AhR into the nucleus of human cells, HepG2 cells were exposed to BPQ or to the classical AhR ligands TCDD and B(a)P, and nuclear extracts were prepared. Incubation of equal amounts of nuclear extracts from untreated cells with a 32P-labeled XRE from the CYP1A1 gene (−983 to −964 bp) failed to induce the formation of a specific AhR-XRE complex. However, nuclear extracts from TCDD- and BPQ-treated cells resulted in the appearance of a specific gel-shifted complex.
This complex was specific for the XRE, because increasing concentrations of cold competitor XRE oligonucleotide successfully eliminated this complex without causing loss of the lower nonspecific band observed in all of the reactions.

As a further confirmation of this result, hepatic cells were treated with DMSO, TCDD, anti-BPDE, or BPQ for 90 min and then fixed for AhR immunofluorescence staining and microscopy (Fig. 10). In untreated cells, all immunofluorescence staining associated with the AhR was present in the cytosol (Fig. 10A), and this staining pattern remained unchanged after treatment with anti-BPDE (Fig. 10C). However, after treatment with either BPQ (Fig. 10D) or the classical AhR ligand TCDD (Fig. 10B), a significant fraction of cells demonstrated intense AhR staining in the nucleus, confirming the ability of BPQ to cause translocation of AhR into the nucleus.

**DISCUSSION**

The initial observation that the proximate carcinogen BP(a)P-diol resulted in a substantial but delayed induction of CYP1A1 mRNA in HepG2 cells implied that a downstream electrophilic metabolite might be responsible for CYP1A1 induction. Coincubation of BP(a)P-diol with two separate AKR inhibitors abolished the induction of CYP1A1 mRNA by BP(a)P-diol, suggesting that the AKR-catalyzed oxidation of BP(a)P-diol to the o-quinone BPQ was the requisite metabolic event. Subsequent studies directly demonstrated that AKR-generated BPQ was nearly equipotent with parent BP(a)P with respect to its ability to induce CYP1A1. To our knowledge, this is the first report of an electrophilic PAH metabolite produced by a phase II (de)toxification enzyme that is capable of inducing a phase I activating enzyme, e.g., CYP1A1, via the AhR pathway.

Because X-ray crystallographic studies show that the oxidation of the nonplanar B(a)P-diol by CYP1A1 results in the even more torsionally strained anti-BPDE (37), it was not surprising that anti-BPDE failed to be an inducer of CYP1A1. In contrast, the oxidation of BP(a)P-diol by AKRs initially results in a catechol, which then undergoes successive one-electron oxidations to form an o-quinone with carbons that are now sp²-hybridized at the 7 and 8 positions, restoring planarity to this electrophilic metabolite. Although there are no crystal structures of PAH o-quinones to date, energy minimization of BP(a)P-diol, anti-BPDE, and BPQ structures using QUANTA/CHARMm verified the uniquely planar nature of BPQ (data not shown), a hallmark of AhR ligands.

These studies definitively show that PAH o-quinones utilize the classical AhR signal transduction pathway to cause the induction of CYP1A1. This was demonstrated by the ability of BPQ to (a) induce CYP1A1 expression over a rapid time course, activate XRE-dependent reporter gene expression, and cause translocation of the AhR to the nucleus; and (b) by the failure of BPQ to induce murine CYP1A1 in AhR- and ARNT-deficient murine hepatoma cells.

We have previously reported that AKR1C1 is constitutively expressed in HepG2 cells and that it oxidizes BP(a)P-diol to BPQ efficiently (12, 30). This would suggest that the induction of CYP1A1 by BP(a)P-diol should occur rapidly and not over the delayed time course reported here. However, a sufficient reservoir of “free” BPQ must first build up to act as a ligand of the AhR. Studies on [³H]BP(a)P-diol metabolism in HepG2 cells reveal that there is a high level of glucuronol- and sulfo-transferase activity present, and that free BPQ is only detected after 12–24 h. Thus, the time required to detect free BPQ after B(a)P-diol treatment is consistent with the time course observed for the indirect induction of CYP1A1 by BP(a)P-diol.

Parent PAHs are classified as bifunctional inducers because as planar molecules they induce CYP1A1 expression via the AhR/XRE pathway, and after metabolism, their downstream metabolites induce phase II (de)toxification enzymes via the ARE pathway (21). However, PAH o-quinones define a novel class of bifunctional inducer that does not require metabolism to induce gene expression via both mechanisms. In previous studies (12, 30), we demonstrated that AKR1C1 is induced (most likely via an ARE/EpRE-type mechanism) by its reaction product, the electrophilic and redox-active BPQ, thereby establishing an autoregulatory loop. PAH o-quinones produced by this pathway are therefore bifunctional because in the

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Fig. 10. BPQ causes translocation of the AhR to the nucleus. Hepa1c1c7 cells were exposed to various treatments for 90 min, and the subcellular distribution of AhR was assessed by indirect immunofluorescence microscopy. A, 1% DMSO; B, 100 nM TCDD; C, 10 μM anti-BPDE; D, 10 μM BPQ.
PAH o-quinones induce CYP via the AhR

A) Classical Bifunctional Inducers (adapted from Prochaska et al., ref 21)

PAH → metabolism → AhR → XRE
CYP1A1 → ARE

B) PAH o-quinones as a New Class of Bifunctional Inducer

PAH-diol → PAH o-quinone → AhR → XRE → CYP1A1

Fig. 11. PAH o-quinones as novel bifunctional inducers. A. PAHs are classical bifunctional inducers that induce phase I enzymes via the AhR and then require CYP-dependent metabolism to electrophilic intermediates to induce phase II enzymes via the ARE (21). B. PAH o-quinones are novel bifunctional inducers that are electrophiles produced by AKR1C1 that can simultaneously induce phase II enzymes via the ARE and phase I enzymes via the AhR without a requirement for further metabolism.

present studies they were also found to induce CYP1A1 via the AhR/XRE pathway. Thus, due to their uniquely dual planar/electrophilic nature, PAH o-quinones possess features that allow them to simultaneously induce both phase I and phase II gene expression through different mechanisms without a requirement for further metabolism. We compare the mechanism of classical bifunctional inducers described by Prochaska et al. (21) with the mechanism of action of the new bifunctional inducers PAH o-quinones (Fig. 11). Because PAH o-quinones can differ in their electrophilicity by several orders of magnitude, their chemical reactivity may determine their relative abilities to activate phase I versus phase II (de)toxification gene expression via the XRE versus the ARE pathways, respectively.

The ability of AKRs to compete with CYPs and thereby divert B(o)P-diol from the potent mutagen and carcinogen anti-BPDE was initially believed to represent a bona fide detoxification route to an innocuous catechol (11). However, it is well established that the resultant catechol spontaneously autoxidizes to BPQ, and that BPQ possesses an undesirable toxicological profile. First, because it is a Michael acceptor, it forms adducts with DNA, RNA, protein, and glutathione. With calf thymus DNA, both stable cG adducts and N7-guanine depurinating adducts have been reported (18, 19). The formation of the adducts proceeds via a ketol that rearranges to a catechol that is subsequently autoxidized to the o-quinone adduct, a sequence that generates reactive oxygen in proximity to DNA. Second, BPQ is redox active and will enter futile cycles to generate ROS multiple times. The amplification of ROS by BPQ and other PAH o-quinones explains their ability to cleave genomic DNA. DNA strand scission proceeds via a Criegee rearrangement and can lead to the formation of base-prophens and malondialdehyde (20). Thus AKR-catalyzed BPQ formation represents a potential alternative promutagenic pathway to the CYP1A1-catalyzed formation of anti-BPDE. However, for these events to occur in whole cells, BPQ must gain access to the nucleus.

Our discovery that PAH o-quinones generated in the cytosol are shuttled into the nuclear compartment via the AhR reveals the only known mechanism by which genotoxic PAH o-quinones can be targeted to the nucleus with specificity. Although higher concentrations of BPQ (20 μM) can be sequestered in a nonspecific manner into the cell pellet, significantly lower concentrations of BPQ (~100–300 nM) result in detectable CYP1A1 induction. Thus, at low concentrations, preferential targeting may indeed be achieved by the AhR. Because BPQ is a redox active compound and will enter into futile cycles, this specific targeting of low concentrations of BPQ could contribute to its genotoxic profile. In this regard, it is well known that after ligand-dependent translocation of the AhR to the nucleus, the AhR is rapidly degraded to limit the duration of CYP1A1 induction (38, 39). It is unclear, however, what happens to the previously bound ligand. Local liberation of PAH o-quinones in proximity to genomic DNA, which is known to contain copper (40, 41), will enhance redox cycling in the presence of nuclear reducing equivalents and provides a mechanism for the oxidative DNA damage observed with parent PAH in vitro and in vivo (42, 43). In the future, it will be interesting to determine whether the genotoxic effects of BPQ are attenuated in AhR-deficient cells.

In addition to the implications for the initiation phase of PAH carcinogenesis, the activation of the AhR by PAH o-quinones may also play a role in the promotion stage. The propensity of PAH o-quinones to redox cycle and amplify ROS in PAH-exposed cells may mimic the tumor-promoting effects of phorbol myristoyl acetate by generating oxidative stress, which can lead to the inappropriate activation of protein kinase C and expression of the proto-oncogenes c-fos and c-jun (44). TCDD is also a well-established tumor promoter (45–48), and the ability of PAH o-quinones to mimic a well-characterized property of TCDD (CYP1A1 induction) may represent an epigenetic property of PAH o-quinones that contributes to the ability of PAHs to act as complete carcinogens.

In summary, these studies demonstrate that PAH o-quinones generated by human AKRs induce the PAH-activating enzyme CYP1A1 via an AhR-dependent mechanism. The ability of genotoxic PAH o-quinones to enter the nucleus and mimic the potent tumor promoter TCDD may have important implications for both the initiation and promotion phases of PAH carcinogenesis. These and other in vitro studies underscore the need to test PAH o-quinones as endogenously generated tumor-initiating/promoting metabolites of PAHs in in vivo models of chemical carcinogenesis.

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REFERENCES

5. Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H., and Jerina, D. M. Metabolism of benzo[a]pyrene: conversion of (Z)-trans-7,8-dihydroxy-7,8-dihydro-


Genotoxic Polycyclic Aromatic Hydrocarbon ortho-Quinones Generated by Aldo-Keto Reductases Induce CYP1A1 via Nuclear Translocation of the Aryl Hydrocarbon Receptor

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