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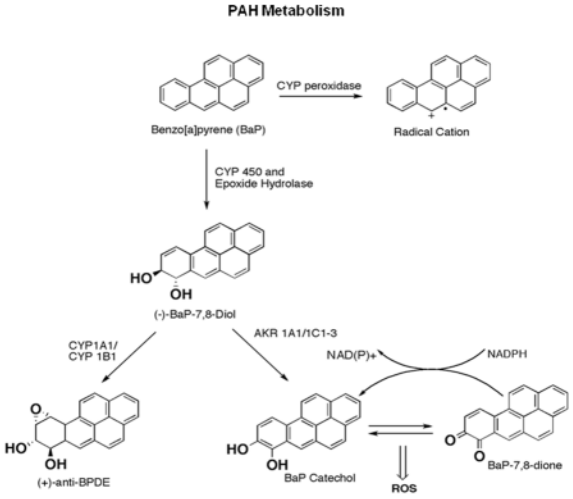
Aldo-keto reductases protect lung adenocarcinoma cells from the acute toxicity of B[*a*]P-7,8-*trans*-dihydrodiol

Zahidur Abedin, **Sushmita Sen**, and **Jeffrey Field**
Department of Pharmacology, Centers for Excellence in Environmental Toxicology and Cancer Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084

Abstract



Tobacco smoke exposure stimulates the expression of genes that are likely to be involved in the metabolism of its combustion products such as polycyclic aromatic hydrocarbons (PAH). Four of the smoke induced genes are aldo-keto reductases (AKR), enzymes that metabolically activate PAH to PAH *o*-quinones. Alternatively, PAHs are metabolized to (±)-*anti*-diol epoxides, such as (±)-*anti*-benzo[*a*]pyrene diol epoxide ((±)-*anti*-BPDE)), by the combined action of P4501A1/1B1 and epoxide hydrolase. (±)-*anti*-BPDE forms DNA adducts directly, while PAH *o*-quinones cause DNA damage by oxidative stress through a futile redox cycle. To address the role of AKRs in PAH cytotoxicity, we compared the cytotoxicity of PAH metabolites and the effects of overexpressing AKR1A1 in lung cells. (±)-*anti*-BPDE and B[*a*]P-7,8-*trans*-dihydrodiol, an intermediate in (±)-*anti*-BPDE metabolism, are toxic to A549 cells at concentrations with an IC_{50} of ~2 μM. In contrast, the PAH *o*-quinone B[*a*]P-7,8-dione was about 10-fold less toxic to A549 cells with an IC_{50} > 20 μM. Similar differences in cytoxicity was observed with two other PAH *o*-quinones (benz[*a*]anthracene-3,4-dione and 7,12-dimethylbenz[*a*]anthracene-3,4-dione) compared with their respective diol-epoxide counterparts (BA-3,4-diol-1,2-epoxide and DMBA-3,4-diol-1,2-epoxide). In addition, both *anti*-BPDE and B[*a*]P-7,8-*trans*-dihydrodiol induced *p53* expression ~6 hours post-treatment at concentrations as low as 1 μM consistent with extensive DNA damage. B[*a*]P-7,8-dione treatment did not induce p53 but generated reactive oxygen species (ROS) in A549 cells and induced the expression of oxidative response genes in H358 cells. We also observed that overexpression of AKR1A1 in H358 cells, which otherwise have low levels of AKR

*To whom correspondence should be addressed: Department of Pharmacology, University of Pennsylvania School of Medicine, 626 CRB, Philadelphia, PA 19104-6084, USA. Tel: 215-898-1912. Fax: 215-573-2236. jfield@upenn.edu.

expression, protected cells 2–10 fold from the toxic effects of B[*a*]P-7,8-*trans*-dihydrodiol. These data suggest that overexpression of AKRs may protect lung cancer cells from the acute toxic effects of PAH.

Keywords

PAH *o*-quinones; Aldo-Keto Reductases; *anti*-BPDE

Introduction

Lung cancer is caused primarily by exposure to tobacco smoke [1]. Tobacco smoke, and other combustion products, such as charbroiled food and automobile exhaust, contains polycyclic aromatic hydrocarbons (PAH) [2]. However, PAH must be metabolically activated into ultimate carcinogens that can damage DNA [3–5]. If left unrepaired, the DNA damage can cause mutations to initiate cancer.

One of the most common PAH in tobacco smoke is Benzo[*a*]Pyrene (B[*a*]P). B[*a*]P can be converted into DNA reactive metabolites via three pathways. The first pathway utilizes enzymes from the cytochrome P450 family (CYP1A1 and CYP1B1) and epoxide hydrolases to form (±)*anti*-BPDE [6]. (±)-*anti*-BPDE is highly mutagenic and forms bulky adducts with DNA [7, 8]. The second pathway utilizes P450 peroxidase to form radical cations which react with DNA to yield depurinating adducts [9, 10]. The third pathway employs aldo-keto reductases (AKRs) to oxidize B[*a*]P-7,8-*trans*-dihydrodiols to catechols which can undergo two spontaneous oxidation events to form PAH *o*-quinones [6]. PAH *o*-quinones can form both stable and depurinating DNA adducts [11, 12]. In addition, during the formation of PAH *o*-quinones, a futile redox cycle occurs in the presence of NADPH, which generates reactive oxygen species (ROS). Based on mutagenesis studies, measurement of ROS levels, and measurements of oxidized macromolecules in cells, it is likely that the redox cycling is the predominant cause of DNA damage by PAH *o*-quinones [13–17].

Efforts to trace the carcinogenic pathways leading to lung cancer have found evidence for all of these mechanisms of PAH activation. The diol epoxide pathway is supported by studies showing that B[*a*]P-7,8-*trans*-dihydrodiol epoxide adducts are found in smokers lungs and the locations of DNA adducts can be mapped to known hotspot codons on the tumor suppressor p53[18]. The PAH *o*-quinone pathway is supported by studies showing that smoking causes oxidative stress, which is characterized by low levels of antioxidants [19] and elevated levels of the oxidative lesion, 8-oxo-2'-deoxyguanosine (8-oxo-dGuo) [20–22]. Expression of AKR1A1 reduced the levels of B[*a*]P-7,8-*trans*-dihydrodiol epoxide adducts in lung epithelial cells, suggesting that AKRs affect the metabolism of PAH [23]. The products of radical cation damage and depurinating adducts have also been reported in PAH treated mice and cells, suggesting radical cations are also generated from tobacco metabolites [10, 23]. Both B[*a*]P-7,8-*trans*-dihydrodiol epoxide adducts [24, 25] and 8-oxo-dGuo can cause G to T transversions [15], the predominant mutation in the tumor suppressor gene *p53* in lung cancer [26, 27].

Microarray studies of the gene expression patterns associated with tobacco smoke exposure found that four AKR isoforms are among the genes that are smoking response genes [28–31]. In one study, three AKR genes were among only nine genes consistently upregulated in smokers when compared with non-smokers and former smokers [30]. Many of the genes induced by tobacco smoke have anti-oxidant response elements in their promoters and are thus likely to be responding to oxidative stress [32]. These expression studies suggest that, by

overexpressing AKRs, smokers divert some of the PAH into o-quinones, reducing the levels of other metabolites.

Another source of PAH is smoky coal, which is widely used for cooking and heating in Xuan Wei County, China. Non-smoking women from this area have elevated rates of lung cancer. Their lung cancers are characterized by G to T transversions in p53 and Ras, but the spectrum of mutations is different from the spectrum of mutations in smokers in that their is one hotspot, codon 249, which is not a preferred PAH adduct site. In addition, within this population there are single nucleotide polymorphisms (SNPs) in AKR1C3 and OGG1 (which repairs 8-oxo-dGuo) which are associated with increased lung cancer risk. These studies suggest a role of AKRs and oxidative damage in smoky coal-induced lung cancer [33, 34].

We hypothesized that AKRs protected cells from exposure to PAH. To address this hypothesis we first tested the toxicity of PAH *o*-quinones to other PAH metabolites in lung carcinoma cells (A549). Next we tested if an intermediate product of PAH metabolism, diols, would be more or less toxic to lung cancer cells that overexpress AKR1A1 (H358/AKR1A1) compared to cells with no AKRs (H358). To determine if cells were undergoing oxidative stress, we stained A549 cells treated with PAH *o*-quinones with a specific dye (H_2DCFDA) used to detect ROS generation. Finally, we examined the expression levels of oxidative response genes in both A549 cells and H358 cells exposed to *o*-quinones to determine if ROS generated by PAH *o*-quinone treatment would induce the expression of oxidative response genes. These results suggest that AKR1A protects lung epithelial cells from the toxic effects of PAH metabolites.

Materials and Methods

Chemicals and Reagents

Reagents and media for cell culture were purchased from Invitrogen (Carlsdbad, CA). B[*a*]P and B[*a*]P metabolites (B[*a*]P-7,8-*trans*-dihydrodiol, (±)-*anti*-BPDE and B[*a*]P-7,8-dione) were obtained from NCI Chemical Carcinogen Standard Reference Repository (Midwest Research Institute, Kansas City, MO). The purity of all PAH-metabolites was assessed by LC/MS. Other reagents 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"*. Ponalrestat (Statil) was purchased from TOCRIS biosciences (Ellisville, MO). B[*a*]P and B[*a*]P metabolites were dissolved in DMSO in accordance with their solubility profiles.

Cell Culture and Toxicity Studies

Human lung adenocarcinoma cells (A549) and human bronchial epithelial cells (H358) were obtained from ATCC (Manassas, VA). A549 cells were cultured in DMEM (Invitrogen Carlsbad, CA) supplemented with 10% FBS and 100 μg/ml Penicillin/Streptomycin. H358 cells were cultured in RPMI 1640 media (Invitrogen Carlsbad, CA) supplemented with 10% FBS and 100 μg/ml Penicillin/Streptomycin. H358/AKR1A1 cells, which overexpress AKR1A1, were obtained from Dr. Trevor Penning (University of Pennsylvania, Philadelphia PA) and grown in RPMI 1640 media supplemented with 10% FBS, 100 μg/mL Penicillin/Streptomycin and Geniticin (G418–0.4 mg/mL). All cells were grown at 37°C in a humidified 6% CO_2 incubator. For toxicity studies, cells were plated at an initial density of 1.0×10^5 cells on 24-well plates, grown for 24 hrs, treated with various P[a]H metabolites and incubated at 37°C for the specified time. Cells were then washed with PBS, trypsinized, stained with trypan blue and counted on a Countess cell counting machine (Invitrogen). All data points represent 3 independent experiments repeated in triplicates.

Western Blot Analysis

Cells were washed with phosphate buffered saline (PBS), trypsinized and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 10% glycerol, 1 mM EDTA pH 8.0, 100 mM NaCl, . 1% SDS, 1% Triton X-100, 1mM sodium orthovanadate, 5 mM sodium fluoride, 1mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor tablet (Roche, Indianapolis, IN). Protein concentration was determined by the Bradford method (Bio-Rad Hercules, CA) and a Nano-drop spectrophotometer (Thermo Scientific West Palm Beach, FL). Samples of extracts containing 25–50 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Billerica, MA). Membranes were next blocked in 5% milk in 1× Tris-Buffered Saline Tween (TBST) for 1 hr and incubated with a specific primary antibody overnight in 5% milk/TBST at a concentration suggested by the manufacturer. Blots were washed in 1 × TBST three times before addition of a secondary antibody [anti-mouse, anti-goat, or anti-rabbit (Sigma)] for 1 hr. Finally blots were washed and bands visualized by enhanced chemiluminescence Western Blot detection kit (GE healthcare, Piscataway, NJ). The following primary antibodies were used (1/1000 dilution): anti-NQO1 sc-16464, anti-HO-1 sc136960, anti-p53 sc-126, anti-Actin sc-1615 (all from Santa Cruz, Santa Cruz, CA), anti-p-p38 4511, anti-p-JNK/SAPK 4668, anti-JNK/SAPK 9258, anti-p38 9212 (all from Cell Signaling, Danvers, MA).

Detection of ROS

Formation of ROS was measured after treating with the fluorescent dye 2',7'-dichlorodihydrofluorescin diacetate (H_2DCFDA) (Invitrogen, Carlsbad, CA). Briefly, 80–90% confluent A549 cells were washed with HBSS twice at 37°C for 5 min. Cells were then pre-incubated with 1 μM of H_2DCFDA dye for 1hr, washed with HBSS and treated as indicated with various concentrations of B[*a*]P-7,8-dione or (±)-*anti*-BPDE. The cells were then incubated for 6 hours at 37°C, washed twice with HBSS 37°C for 5 min each time and observed under a fluorescent microscope to determine ROS generation (Nikon Eclipse T5100).

Realtime PCR and PCR arrays

Real-time quantitative reverse transcription and polymerase chain reaction was performed using the taq-man assay (AB Step One Plus, Applied Biosystems, Germany). The probes and primers were acquired from Applied Biosystems and are as follows: human NQO1 (Hs00168547_m1), HO-1 (Hs01110251_m1) and GAPDH (Hs99999905_m1). Each experiment was standardized with GAPDH primers as an internal control and consisted of three samples in triplicates. RT-PCR array was performed using the human oxidative stress and antioxidant defense kit from SA biosciences (Fredkerick, MD).

Statistical Analysis

Results are presented as mean ± standard error of the mean. The student t-test was used to determine p-values between samples.

Results

PAH *o*-quinones are less cytotoxic to lung epithelial cells than (±)-*anti*-BPDE and B[*a*]P-7,8-*trans*-dihydrodiol

To address the role of AKRs in lung cell viability, we first compared the toxicity of the AKR metabolites, PAH *o*-quinones, with other PAH metabolites in lung cells. We treated A549 cells with the metabolites and measured cell viability using the trypan blue exclusion method. (±)-*anti*-BPDE and B[*a*]P-7,8-*trans*-dihydrodiol were cytotoxic as shown in

previous studies [35]. The IC_{50} for both (±)-*anti*-BPDE and B[*a*]P-7,8- *trans*-dihydrodiol was ~ 2.0 μM at 24 hr (Fig. 1A). In contrast, all concentrations of the PAH *o*-quinone B[*a*]P-7,8-dione (BPQ), including concentrations near the limits of solubility (20 μM), reduced cell viability by ≤ 20% at 24 hours (Fig 1A) and this reduction was not statistically significant. To further evaluate the level of toxicity of the metabolites, we increased the period of treatment. There was virtually no cell death in A549 cells treated with B[*a*]P-7,8-dione up to 72 hrs of exposure compared to DMSO alone (Fig. 1D). At 5μM of (±)*anti*-BPDE, however, ≥80% of the cells died by 24 hr (Fig. 1D). Thus, B[*a*]P-7,8-dione is significantly less toxic to A549 cells than other PAH metabolites (±)-*anti*-BPDE and B[*a*]P-7,8-*trans*-dihydrodiol. To address the relative toxicity of other quinone/diol-epoxide pairs, we tested benz[*a*]anthracene-3,4-dione (BAQ) and BA-3,4-diol-1,2-epoxide (BADE) as well as 7,12-dimethylbenz[*a*]anthracene-3,4-dione (DMBAQ) and DMBA-3,4-diol-1,2-epoxide (DMBADE). In each pair we found that the quinone was significantly less cytotoxic than its diol epoxide (Fig 1B–C). We conclude that that PAH *o*-quinone metabolic activation pathways are less cytotoxic than diol-epoxide pathways.

Overexpression of AKRs protect bronchial alveolar cells (H358) from the toxic effects of Bp 7,8 diol

To test the hypothesis that AKRs are metabolizing PAH to less toxic metabolites, we tested cells that overexpress AKRs. Because A549 cells express multiple AKR isoforms at high levels, it was not possible to manipulate the levels of AKRs in them. However, H358 cells express low levels of AKRS and can be engineered to express high levels of AKR1A1 and convert B[*a*]P-7,8-*trans*-dihydrodiols into PAH *o*-quinones[36]. Again, as in the A549 cells, we found that the PAH *o*-quinones were significantly less toxic than B[*a*]P-7,8-*trans*-dihydrodiol or (±)-*anti*-BPDE in H358 cells (Figure 2A). However, the cells that overexpress the AKR1A1 (H358/AKR1A1) were resistant to B[*a*]P-7,8- *trans*-dihydrodiol (Fig. 2B) suggesting that AKR1A1 was converting the B[*a*]P-7,8- *trans*-dihydrodiol to the less cytotoxic PAH *o*-quinones. To confirm that resistance was the result of AKR overexpression, we performed the toxicity studies in the presence of an AKR1A1 inhibitor, Statil. Treating cells with 50 and 100 μM of Statil reduced cell viability by 35 and 55 percent, respectively, enhancing the toxicity of B[*a*]P-7,8-*trans*-dihydrodiol (Fig. 2C). The viability of the treated cells was about the same as those that do not overexpress AKR1A1. These concentrations of statil did not affect viability of cells in the absence of B[*a*]P-7,8-*trans*-dihydrodiol. Collectively, these results support the hypothesis that overexpression of AKRs protect lung cancer cell lines from the toxic effects of B[*a*]P-7,8-*trans*-dihydrodiol.

Response of Stress genes to PAH metabolites

We next determined if B[*a*]P-7,8-dione induced stress responses in cells. We first tested p53 induction, which is induced by DNA damage. p53 expression was stimulated in A549 cells exposed to 1 μM of (±)*anti*-BPDE for 6 hrs as well as 24 hrs (Fig. 3A). B[*a*]P induced p53 expression but with delayed kinetics compared to (±)*anti*-BPDE. This delay probably reflects the time required for B[*a*]P to be converted into DNA reactive metabolites. However, B[*a*]P-7,8-dione did not stimulate p53 expression even at concentrations up to 20 μM (Fig. 3A). To confirm that B[*a*]P-7,8-dione generated ROS, we pre-incubated A549 cells with 2',7'-dichlorodihydrofluorescin diacetate (H_2DCFDA), a fluorescent marker that detects ROS, and treated A549 cells with 20uM B[*a*]P-7,8-dione for 6 hrs. B[*a*]P-7,8-dione generated high levels of ROS, as visualized by the increased fluorescent intensity, while (±)-*anti*-BPDE treated cells did not generate ROS (Fig. 3B).

We also examined other markers of stress, including phosphorylation of p38 and JNK/SAPK. The phosphorylation levels of both were elevated with (±)*anti*-BPDE and B[*a*]P-7,8-*trans*-dihydrodiol (Fig. 3C). However both p-p38 and p-JNK/SAPK showed little change

with B[*a*]P-7,8-dione treatment. Similar results were observed in p-p38 and p-JNK/SAPK in H358 cells (data not shown). Together these experiments confirm that PAH *o*-quinones stimulate ROS, but interestingly do not induce double stranded breaks, which are required to induce p53 [37, 38].

Induction of oxidative response genes by B[*a*]P-7,8-dione

Oxidative stress can be measured by examining the relative expression of a set of genes that have been determined to respond to various types of oxidative stress. To determine which oxidative response genes were elevated due to the exposure of *o*-quinones, we performed a PCR array on H358 cells treated with PAH *o*-quinones. A total of 84 genes were analyzed on an array. The highest level of induction was 3.4 fold for Sulfiredoxin 1 homolog (SRNX1-Data not shown).

We also analyzed two widely studied genes, NAD(P)H quinone oxidoreductase 1 (*NQO1*) and Heme Oxygenase 1 (*HO-1*) as markers for oxidative stress, individually, because they were not represented on the array. We analyzed the genes in both A549 and H358 cell lines. Using Real-Time-PCR on mRNA isolated from A549 cells treated with B[*a*]P-7,8-dione, we found that at 6 hrs *NQO1* expression levels do not significantly change between control group (DMSO only) and treatment group (B[*a*]P-7,8-dione) (Fig. 4A). However, at 24 hrs, the expression of *NQO1* and *HO-1* mRNA levels were elevated by ~2 fold ($p<.05$ for *NQO1*; $p<.005$ for *HO-1*) (Fig. 4A,C). This suggests that oxidative response genes are upregulated in A549 cells after B[*a*]P-7,8-dione exposure. We also observed that NQO1 protein expression was also elevated relative to DMSO control in A549 cells after B[*a*]P-7,8-dione exposure (Fig 4B). These data suggest that treating A549 cells with B[a]P-7,8-dione induces the expression of oxidative response genes, which may be involved in cell protection.

Induction of HO-1 in H358 cells exposed to B[*a*]P-7,8-dione

A549 cells do not respond well to oxidative stress because they harbor a mutation in Kelch-like ECH-associated protein 1 (*KEAP1*), a negative regulator of oxidative response genes[39, 40]. As a result, these cells have elevated levels of oxidative response genes, which are only weakly stimulated by oxidative stress. Therefore we conducted additional stress studies in H358 cells. Note that H358 cells are *p53* negative so we focused on oxidative stress response genes. Quantitative real-time PCR showed that *HO-1* mRNA expression was elevated 20 fold in the H358 cells (Fig. 5A; $p<.001$) and 85 fold in H358/AKR1A1 cells (Fig. 5C; $p<.001$) in response to 20 μM of B[*a*]P-7,8-dione. In addition we also observed a concentration dependent increase in HO-1 protein levels exposed to 5, 10 and 20 μM of B[*a*]P-7,8-dione in both H358 (Fig. 5B) cells and H358/AKR1A1 cells (Fig. 5D). Interestingly, B[*a*]P-7,8-*trans*-dihydrodiol did not induce an increase of HO-1 induction up to 20 μM.

Induction HO-1 by PAH *o*-quinones is ROS dependent

To address if quinone induction of HO-1 is dependent on ROS, we treated H358/AKR1A1 cells with the anti-oxidants α-tocopherol (Vitamin E) or ascorbic acid (Vitamin C) (Fig. 6) prior to quinone induction. Both compounds prevented HO-1 induction, suggesting that B[*a*]P-7,8-dione induces HO-1 through ROS.

Discussion

Smokers, and mice exposed to smoke, are under oxidative stress resulting in reduced levels of plasma antioxidants, increased expression of oxidative response genes and elevated levels of 8-oxo-dGuo in their DNA and in their urine [20–22, 41, 42]. One source of oxidants are

quinones, which can be generated by combustion or alternatively when PAH are metabolized by AKRs. The *o*-quinones are not mutagenic by themselves, but become highly mutagenic in the presence of NADPH because they undergo redox cycling to generate ROS [14, 43–45]. ROS oxidizes DNA to cause 8-oxo-dGuo, a mutagenic lesion that is misread to cause G to T transversions, which are characteristic of tobacco induced lung cancers [14–16, 46, 47].

Microarray analysis identified genes that are induced in the epithelia of smokers, the oral mucosa of smokers and in cells that are exposed to cigarette smoke condensate [28–31]. While multiple studies found AKRs induced, the study from Zhang et. al., also examined former smokers and non-smokers and found that nine genes were induced in smokers but not in former smokers or non-smokers [30]. These genes were: *ALDH3A1*, *CYP1B1*, *MUC5AC*, *NQO1* and *SCGB1A1*. Most of the induced genes are likely to have protective roles. ALDH3A1 (aldehyde dehydrogenase), CYP1B1, NQO1 (NAD(P)H quinone oxidoreductase 1), AKR1C2, AKR1B10, AKR1C1 and AKR1C3 all metabolize tobacco combustion products [31] while *MUC5AC*, encodes mucin, a major component of mucus. There are some direct examples of these genes having protective roles. For example, *NQO1* knockout mice are more susceptible to the toxic effects of quinones [48]. Although knockouts of *CYP1B1* and *CYP1A1* found some unexpected toxicities, the outcomes suggest that CYP1B1 and CYP1A1 both metabolize benzopyrene, depending on expression levels of each in different tissues [49]. Since PAH are metabolized by AKRs into PAH *o*-quinones, we addressed the role of AKRs by overexpression, as well as by comparing the toxicity of PAH *o*-quinones relative to other PAH metabolites.

We hypothesized that AKRs are part of the protective response to PAH induced stress. We measured genotoxic stress by p53 induction, oxidative stress by ROS generation, and induction of oxidative response genes. As documented previously, we found that PAH diol-epoxides ((±)-*anti*-BPDE) efficiently induced genotoxic stress while PAH *o*-quinones showed signs of oxidative stress[32, 50]. Perhaps most striking was the observation that PAH *o*-quinones were not cytotoxic despite evidence of oxidative stress and induction of *HO-1*. Overexpression of AKRs in a cell that expresses low levels of AKRs also reduced the toxicity of the B[*a*]P-7,8-*trans*-dihydrodiols. Together these data suggest a protective role for AKRs in response to PAH exposure.

We propose that cells expressing higher levels of AKRs direct a portion of the PAH from being metabolized into the more toxic (±)-*anti*-BPDE. In adduct studies, when H358 cells were treated with (±)-B[*a*]P-7,8-dihydrodiol, (±)-*anti*- *trans*-B[*a*]P DE-N2-dGuo was the major DNA adduct observed. However, overexpression of AKR1A1 reduced the levels of this DNA adduct from 46 ± 4 adducts/10^6 bases to 26 ± 2 adducts/10^6 bases. Thus AKR1A1 prevents the accumulation of diol epoxide DNA adducts, most likely by converting diols into other metabolites such as B[*a*]P-7,8-dione [51]. In the *CYP1B1* and *CYP1A1* knockout mice studies, adduct levels also correlated with toxicity [49]. These adduct studies support our cytotoxicity observations, since we find that (±)-*anti*-BPDE, which forms (±)-*anti*- *trans*-B[*a*]PDE-N2-dGuo adducts is more cytotoxic than B[*a*]P-7,8-dione.

Although B[*a*]P-7,8-dione stimulated ROS in A549 cells, it did not stimulate p53 expression. This may be because the ROS generated by B[*a*]P-7,8-dione exposure does not cause enough double strand breaks, which are required to induce p53 [37]. ROS damages DNA through formation of oxidation products such as 8-oxo-deoxyguanosine, while (±)-*anti*-BPDE forms bulky adducts. The (±)-*anti*-BPDE adducts are probably causing double-stranded breaks, perhaps through the extensive activation of repair pathways.

While we observed a strong induction of *HO-1* in H358 cells, we found high constitutive levels of *HO-1* expression and only a small induction in A549 cells. *HO-1* transcription is activated by the transcription factor NRF-2, which upon oxidation is released by the inhibitory factor KEAP1. A549 cells have a mutation in *KEAP-1*, which leads to the high constitutive levels of NRF-2 leading to high levels of *HO-1* and other oxidative response genes [39, 40].

We note that while the short term effects of AKR expression may be protective to cells, AKR derived PAH *o*-quinones can undergo futile redox cycling to generate ROS, which is likely to cause oxidative stress. Although we did not detect induction of HO-1 from B[*a*]P-7,8-*trans*-dihydrodiol treatment, oxidatively damaged DNA has been reported in A549 cells [50]. The differences may be due to the expression of different isoforms and differences in the oxidative response genes between the two cell lines.

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Abbreviations

(±)-anti-BPDE	(±)anti-benzo[*a*]pyrene 7,8-diol 9,10-epoxide
BP-7,8-diol or Diol	B[*a*]P-7,8-trans-dihydrodiol
BPQ	benzo[*a*]pyrene-7,8-dione
BAQ	benz[*a*]anthracene-3,4-dione
DMBAQ	7,12-dimethylbenz[*a*]anthracene-3,4-dione
BADE	benz[*a*]anthracene-3,4-diol-1,2-epoxide
DMBADE	7,12-dimethylbenz[*a*]anthracene-3,4-diol-1,2-epoxide
PAH	polycyclic aromatic hydrocarbons
H_2DCFDA	2',7'-dichlorodihydrofluorescin diacetate

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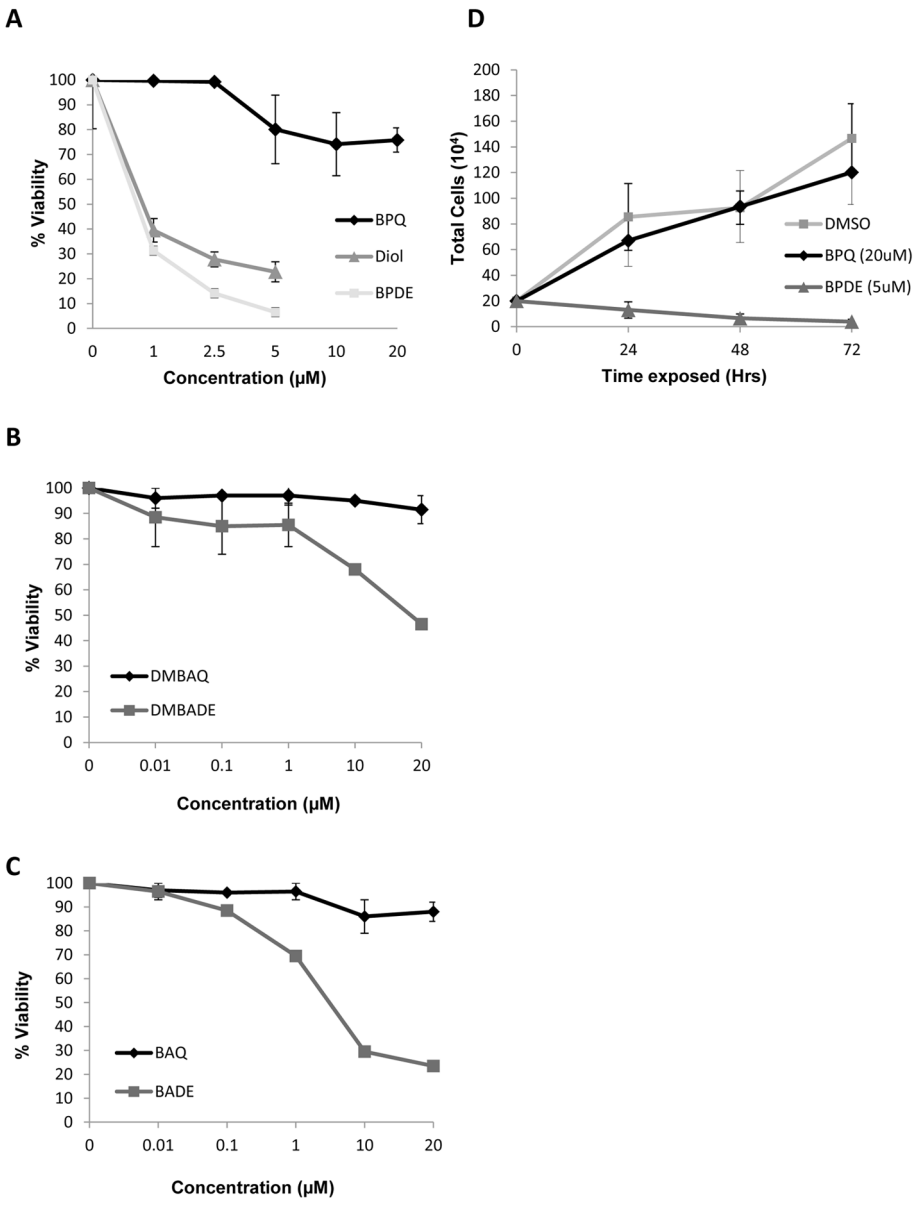

Figure 1.

PAH *o*-quinones are less cytotoxic than PAH diols and PAH diol epoxides to A549 cells. A) A549 cells were seeded at density of 1.0×10^5 cells per well and allowed to attach for 24 hrs. Cells were then treated with varying concentrations of anti-BPDE, B[*a*]P-7,8-*trans*-dihydrodiol (Diol) and B[*a*]P-7,8-dione (BPQ) for 24 hrs, trypsinized and counted using the trypan blue exclusion method. B–C) Cell viability of A549 cells treated with (B) DMBAQ and DMBADE (C) BAQ and BADE for 24 hrs. D) A549 cells were treated with different concentrations of BPQ and anti-BPDE and allowed to grow for 72 hours. At each time point cells were trypsinized and counted using trypan blue exclusion method. Results are the mean of three independent experiments.

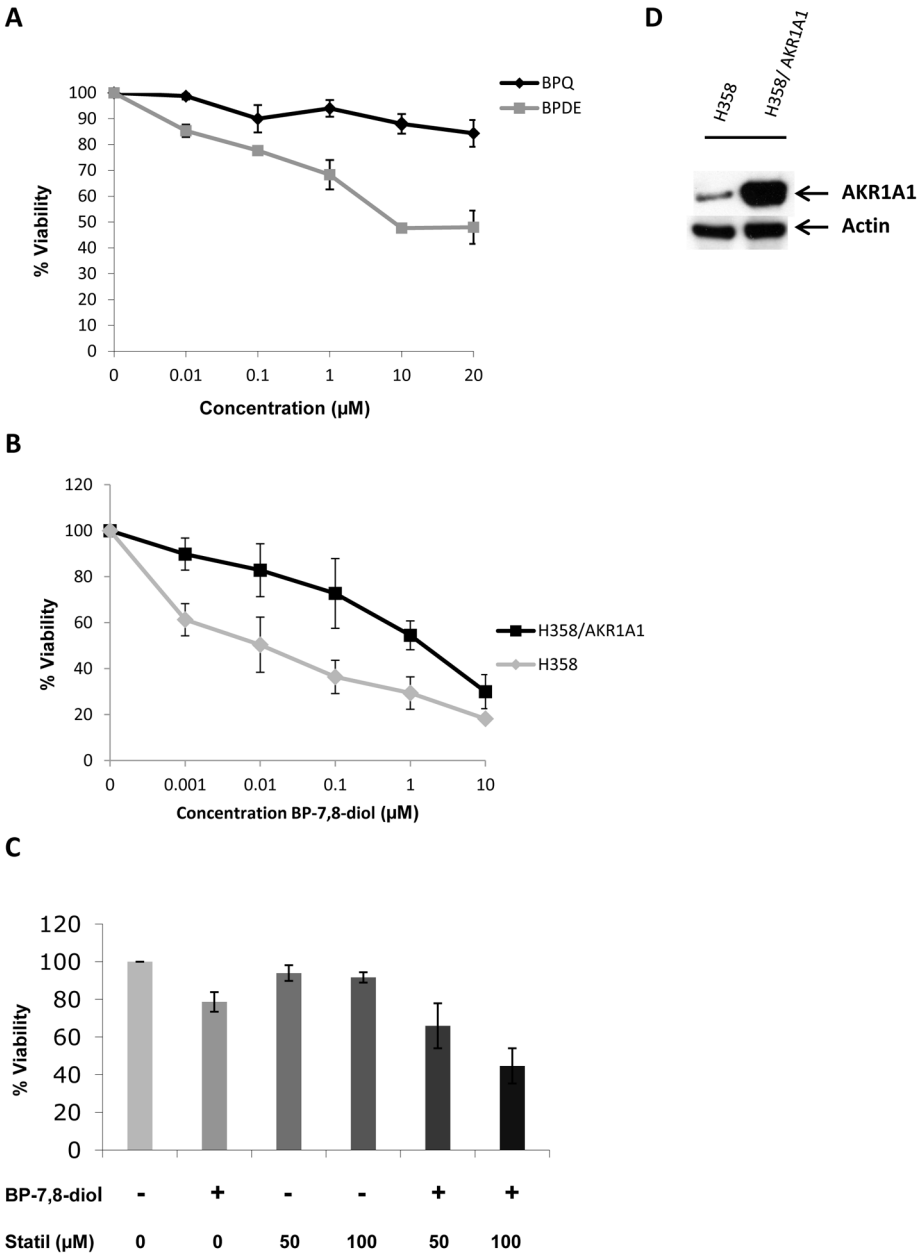

Figure 2.

AKR1A1 overexpression protects lung cells from the toxic effects of PAH metabolites at the expense of increased oxidative stress. A) H358 cells were treated with various concentrations BPQ and anti-BPDE, grown for 24 hrs and cell viability determined using the trypan blue exclusion method. B) Cell viability of H358 and H358/AKR1A1 cells treated with various concentrations of B[*a*]P-7,8-*trans*-dihydrodiol for 24 hrs. C) H358/AKR1A1 cells were treated with B[*a*]P-7,8-*trans*-dihydrodiol and an AKR1A1 inhibitor (statil) for 24 hrs and cell viability determined. D) Western blot analysis showing overexpression of AKR1A1 in H358/AKR1A1 cells compared to H358 cells.

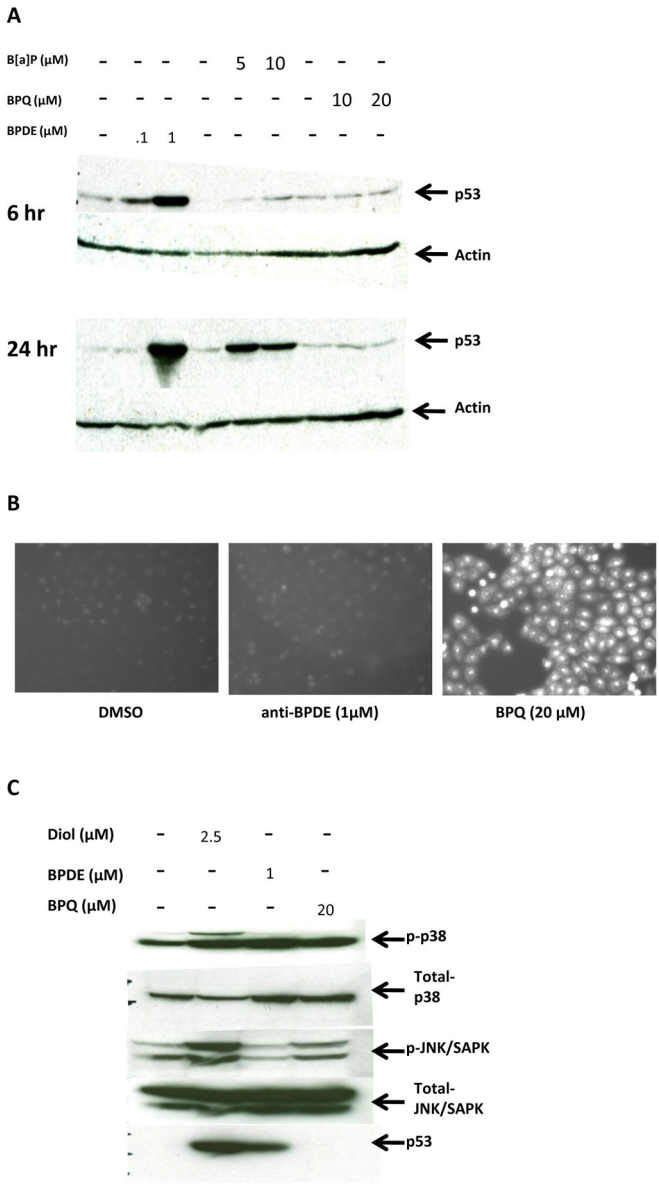

Figure 3.

Response of Stress genes to PAH metabolites. A) A549 cells were treated with B[*a*]P, anti-BPDE or BPQ for 6 hours or 24 hours. Western analysis was performed on A549 total cell lysates with anti-p53 antibodies. B) A549 cells were incubated with H_2DCFDA for 1 hr, washed with HBSS and treated for 6 hrs with DMSO, *anti*-BPDE or BPQ. Cells were then visualized under a fluorescence microscope and images documented. C) Western analysis using stress kinase antibodies was performed on A549 cells treated with DMSO, B[*a*]P-7,8-*trans*-dihydrodiol, *anti*-BPDE, or BPQ for 24 hrs.

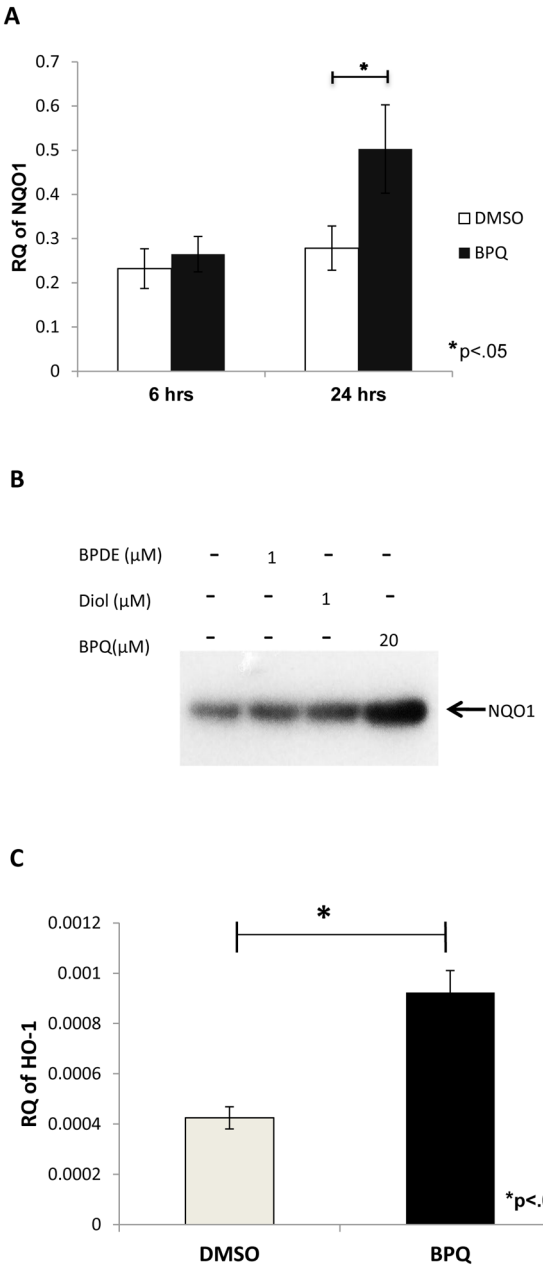

Figure 4.

Increase of *NQO1* and *HO-1* mRNA levels in A549 cells treated with BPQ. A) Realtime PCR showing expression levels of *NQO1* mRNA in A549 cells treated with BPQ for 6 hrs and 24hrs. C) Western blot analysis for NQO1 in A549 cells treated with PAH metabolites. D) Realtime PCR showing increase in *HO-1* mRNA levels isolated from A549 cells treated with B[*a*]P-7,8-dione for 24 hrs. All realtime PCR experiments were performed with 3 biological replicates in triplicates for each sample and standardized to GAPDH expression levels.

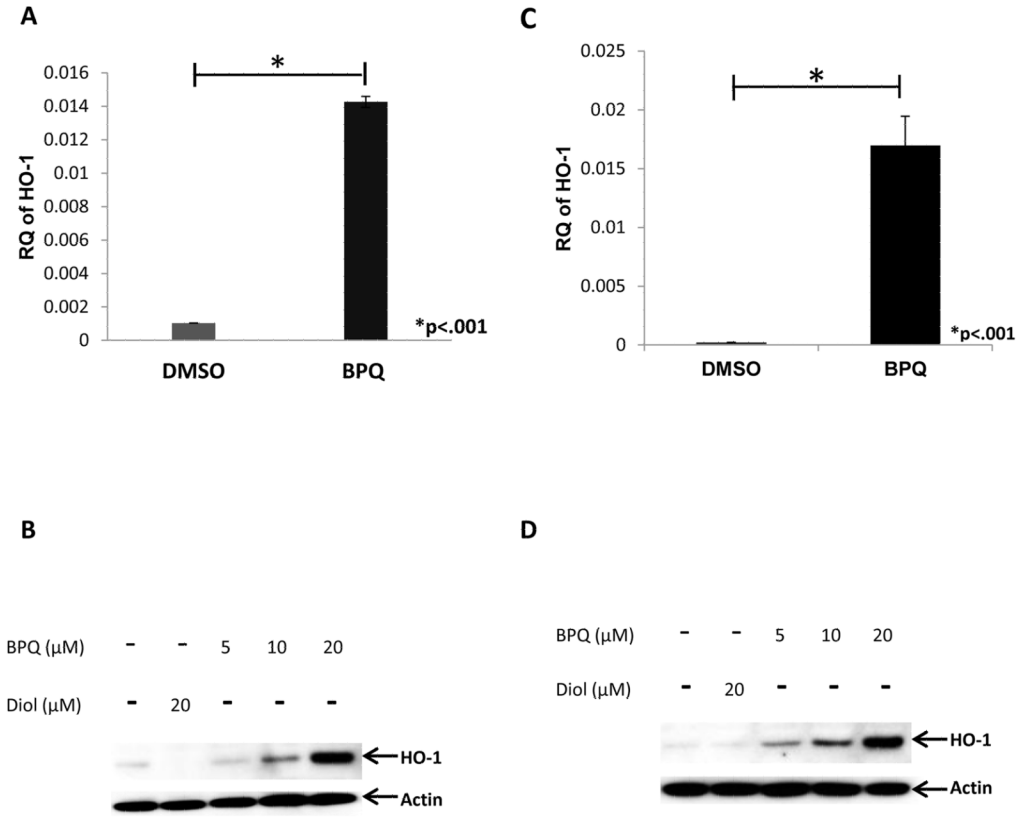

Figure 5.

Increased expression of *HO-1* mRNA in H358 cells and H358/AKR1A1 cells exposed to BPQ. A) Realtime PCR reveals highly elevated levels of *HO-1* mRNA in H358 cells exposed to BPQ for 24 hrs. B) Dose dependent increase of HO-1 protein expression in H358 cells exposed to increasing concentrations of BPQ C) Increase of *HO-1* mRNA levels in H358/AKR1A1 cells exposed to BPQ for 24 hrs. D) Increase in HO-1 protein levels in H358/AKR1A1 cells exposed to increasing concentration of BPQ for 24 hrs.

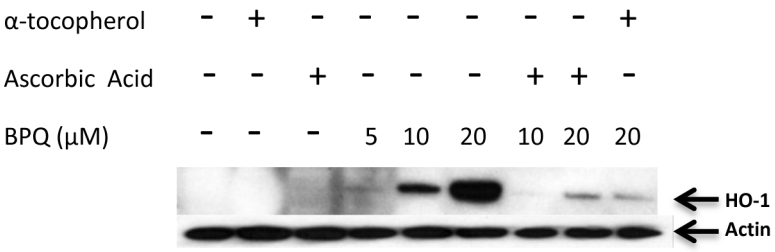

Figure 6.
Antioxidants abate HO-1 induction in H358/AKR1A1 cells treated with BPQ. Western blot analysis for HO-1 on H358/AKR1A1 cells exposed to BPQ and 250 μM of α-tocopherol and ascorbic acid.