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Reduction of androgen receptor expression by benzo[*a*]pyrene and 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in human lung cells

Pinpin Lin*, Jinghua Tsai Chang, Jiunn-Liang Ko, Sheng-Hung Liao, Wai-Sze Lo

Institute of Toxicology, Chung Shan Medical University, No. 110, Sector 1, Chien-Kuo N. Rd., Taichung 40 203, Taiwan, ROC

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Abstract

5α-Dihydrotestosterone significantly increased cell growth of lung adenocarcinoma cell line H1355. Benzo[*a*]pyrene (BaP) was a pulmonary carcinogen found in cigarette smoke. Treatment with 1 μM BaP tremendously reduced constitutive androgen receptor (AR) expression, as determined with Western immunoblotting and the real-time RT–PCR assay, as well as testosterone-induced AR protein levels in H1355 cells. Similarly, 1 μM BaP significantly reduced *AR* mRNA levels in human bronchial epithelial cells BEAS-2B. Although BaP, 2,3,7,8-tetrachlorodibenzo-*p*-dixin and polychlorinated biphenyl 126 activated aryl hydrocarbon receptor (AhR), which subsequently induced *cytochrome P4501A1* (*CYP1A1*) and *P4501B1* (*CYP1B1*) expression in H1355 cells, unexpectedly, neither TCDD nor PCB126 reduced AR expression. Antagonizing AhR activation and cytochrome P4501 activity with α-naphthoflavone, or inhibiting CYP1B1 activity with 2,4,3′,5′-tetramethoxystilbene, however, prevented BaP-induced AR reduction. Furthermore, 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, a BaP carcinogenic metabolite catalyzed by CYP1A1 and CYP1B1, significantly reduced AR expression in H1355 cells and human lung fibroblasts WI-38. This was the first study that reports that BaP and BPDE reduced endogenous *AR* expression. These data suggest that metabolically activated BaP may disrupt androgen function by reducing AR levels in androgen-responsive organs.

Keywords: Androgen receptor; Benzo[*a*]pyrene; Cytochrome P4501A1; Cytochrome P4501B1; Lung cells

1. Introduction

Exposure to polycyclic aromatic hydrocarbons (PAH)-contaminated air pollutants has been associated with the occurrence of pulmonary diseases [1]. Benzo[*a*]pyrene (BaP), found in cigarette smoke and air pollutants, is one of the most widely studied PAH [2,3]. It is well recognized that BaP activates the aryl hydrocarbon receptor (AhR) which subsequently induces *cytochrome P4501A1* (*CYP1A1*) and *P4501B1* (*CYP1B1*) expression [4]. *CYP1A1* and *CYP1B1* are involved in the conversion of BaP into 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), which forms DNA adducts [5,6]. CYP1B1 also converts BaP into other ultimate metabolites, such as 3-hydroxy benzo[*a*]pyrene(3OHBaP), which attack cellular DNA [7]. Several lines of evidences have shown that BaP-induced genotoxicity and carcinogenesis are AhR-dependent [8,9]. AhR belongs to one of the nuclear receptor superfamilies. While shared some regulatory proteins with steroid hormone receptors [10,11], AhR was reported to interact with several steroid hormone receptors, including estrogen receptor, androgen receptor (AR), glucocorticoid receptor and progesterone receptor, to alter their functions [12–14]. Therefore, BaP may activate AhR and subsequently interfere with steroid hormone receptors function to elicit toxicological responses.

Most studies have investigated effects of sex hormones on steroidogenic and steroid hormone-responsive organs, such as the reproductive system. However, growing evi-

* Corresponding author. Tel.: +886-4-24730022; fax: +886-4-24751101.
E-mail address: ppl@csmu.edu.tw (P. Lin).

Abbreviations: BaP, benzo[*a*]pyrene; AR, androgen receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dixin; PCB126, polychlorinated biphenyl 126; AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P4501A1; CYP1B1, cytochrome P4501B1; BPDE, 7,8-dihydro-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbons; DHT, 5α-dihydrotestosterone; ANF, α-naphthoflavone; TMS, 2,4,3′,5′-tetramethoxystilbene; RT–PCR, real-time reverse transcription–polymerase chain reaction.

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dences show that sex hormones also target non-genital organs. Several studies reported that AR is expressed in normal fetal and adult lungs and human lung tumors [15,16]. Testosterone and 5α-dihydrotestosterone (DHT) have been shown to affect pulmonary surfactant production during fetal lung development [17,18]. More recently, Ojeda *et al.* [19] demonstrated that surgical and pharmacological castration significantly modified the components of phospholipids in lung surfactant of adult male rats. This alteration was similar to that observed in human lung emphysema. These information suggests that testosterone is required to maintain lung morphology. Therefore, exposure to antiandrogens may have adverse effects on normal lung morphology and function.

The effects of PAH on androgen function are rarely reported. It is well-known that the androgenic effect is dependent on AR activation. Vinggaard *et al.* [20] showed that PAH, including BaP, antagonized recombinant human AR activity in a the reporter gene assay system. Kizu *et al.* [21] reported that PAH antagonized the DHT-induced expression of prostate-specific antigen in LNCaP human prostate carcinoma cells. Both Vinggaard and Kizu's studies demonstrated that AhR was required for the anti-androgenic effect of PAH [20,21]. In our present study, we examined effect of BaP in AR protein and gene expression in human lung cells. We further investigated how the AhR signaling pathway is involved in the effect of BaP on AR expression.

2. Materials and methods

2.1. Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyl 126 (PCB126) were purchased from ULTRA Scientific. α-Naphthoflavone (ANF), BaP, dimethylsulfoxide, DHT and testosterone were purchased from Sigma. BPDE was purchased from Midwest Research Institute. 2,4,3′,5′-Tetramethoxystilbene (TMS) was ordered from Tocris Cookson Inc. RPMI 1640, Eagle's basal medium, and fetal bovine serum were purchased from Gibco™ Invitrogen Inc.

2.2. Cell culture

The human lung adenocarcinoma cell lines NCI-H1355 were gifts from Dr. C.-M. Tsai (Veterans General Hospital, Taipei, Taiwan, ROC). Information for H1355 was available in the American Cell Type Cell Collection. H1355 cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Human lung fibroblasts WI-38 were purchased from the American Type Culture Collection and maintained in Eagle's basal medium containing 10% fetal bovine serum. BEAS-2B cells were human bronchial epithelial cell lines immortalized with SV40 (American Type Culture Collection) and maintained in LHC-9 medium (BioSource International Inc.).

2.3. Cell viability assay

H1355 cells were seeded to 12-well plates. Twenty-four hours later, the medium was changed to phenol red-free medium containing 5–10% charcoal-stripped fetal bovine serum and incubated for another 48 hr. After treatment with DHT for 6 days, cell viability was determined with trypan blue exclusion assay.

2.4. Quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) assay

The total RNA of the cells was isolated using the TRIZOL® Reagent (Life Technologies) and the phenol–chloroform extraction method. Synthesis of cDNA was done with 2 μg total RNA, 1 μg oligo dT primer and 20 nmol deoxynucleotide triphosphates using M-MLV Reverse Transcriptase (Promega). Quantitative PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) and analyzed on an ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystem). Primers were chosen with the assistance of the computer program—Primer Express (Perkin-Elmer Applied Biosystem). The primer sequences and optimal concentrations of *CYP1A1*, *CYP1B1*, *AhR*, *AR* and *β-actin* were previously described [22,23]. Each data point was repeated three times. Quantitative values were obtained from the threshold PCR cycle number (Ct), where the increase in signal associated with an exponential growth for PCR product becomes detectable. The relative mRNA levels in each sample were normalized to its *β-actin* content. The relative expression levels of target gene $= 2^{-\Delta Ct}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$.

2.5. Western immunoblotting

Cells were sonicated in 20 mM Tris pH 7.4, 0.5 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 50 μg/mL leupeptin, 50 μg/mL aprotinin, 2.5 μg/mL pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors cocktail (Roche Applied Science). Cell homogenates were centrifuged at 10,000 *g*, 4°. The cytosol supernatants were dissolved in sample buffer for electrophoresis. The cytosol in sample buffer were boiled, resolved by a denaturing electrophoresis on discontinuous polyacrylamide gel, electrotransferred to a PVDF membrane and immunostained with AR antibody (Upstate Co). The bands were visualized with the enhanced chemoluminescence kit according to the manufacturer's instructions (Amersham Biosciences). Band intensity was quantified with the ChemiImager™ System (Alpha Innotech Corp.). The relative expression of AR protein was calculated by normalizing to the band intensity of β-actin.

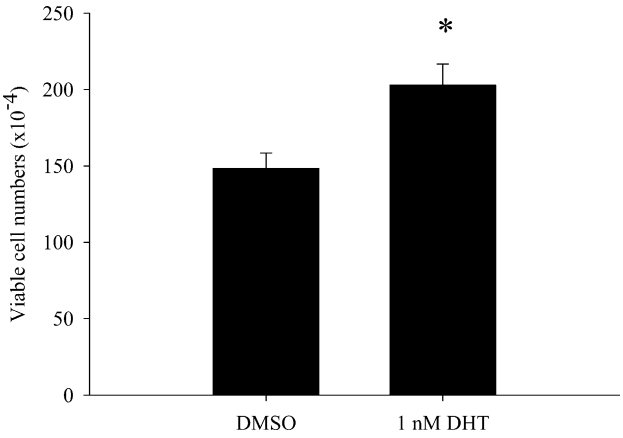

Fig. 1. Effect of DHT on the cell growth of H1355 cells. H1355 cells were treated with 0.1% DMSO or 1 nM DHT for 6 days. Viable cell numbers were determined with the trypan blue exclusion assay. Each data is the mean of four replicates. The symbol (*) indicates $P < 0.05$ as comparing with DMSO-treated cells.

2.6. Statistical analysis

Comparisons of the cell numbers, quantification of Western immunoblotting or gene expression levels between groups were done by Student's *t* test.

3. Results

3.1. Effects of BaP on constitutive or testosterone-induced AR expression in lung cells

When human lung adenocarcinoma cells H1355 were treated with 1 nM DHT, viable cell numbers were significantly increased 6 days later (Fig. 1). This result indicated that the H1355 cells were responsive to androgens. Treatment with 1 or 10 μM BaP slightly but significantly reduced AR protein levels in H1355 cells 24 hr later (Fig. 2A). The reduction became more dramatic 72 hr later (Fig. 2A). Furthermore, *AR* mRNA levels in H1355 and BEAS-2B cells were significantly reduced by 1 μM BaP 48 or 72 hr later (Fig. 2B and C). Therefore, the decrease in *AR* mRNA levels might partially count for the decrease in AR protein levels by 1 μM BaP treatment. While AR protein levels were significantly increased by 10 nM testosterone in H1355 cells (Fig. 3), co-treatment with BaP prevented the increase in AR levels by testosterone (Fig. 3). These results indicated that BaP reduced the AR expression in H1355 and BEAS-2B cells at the transcription and translation levels.

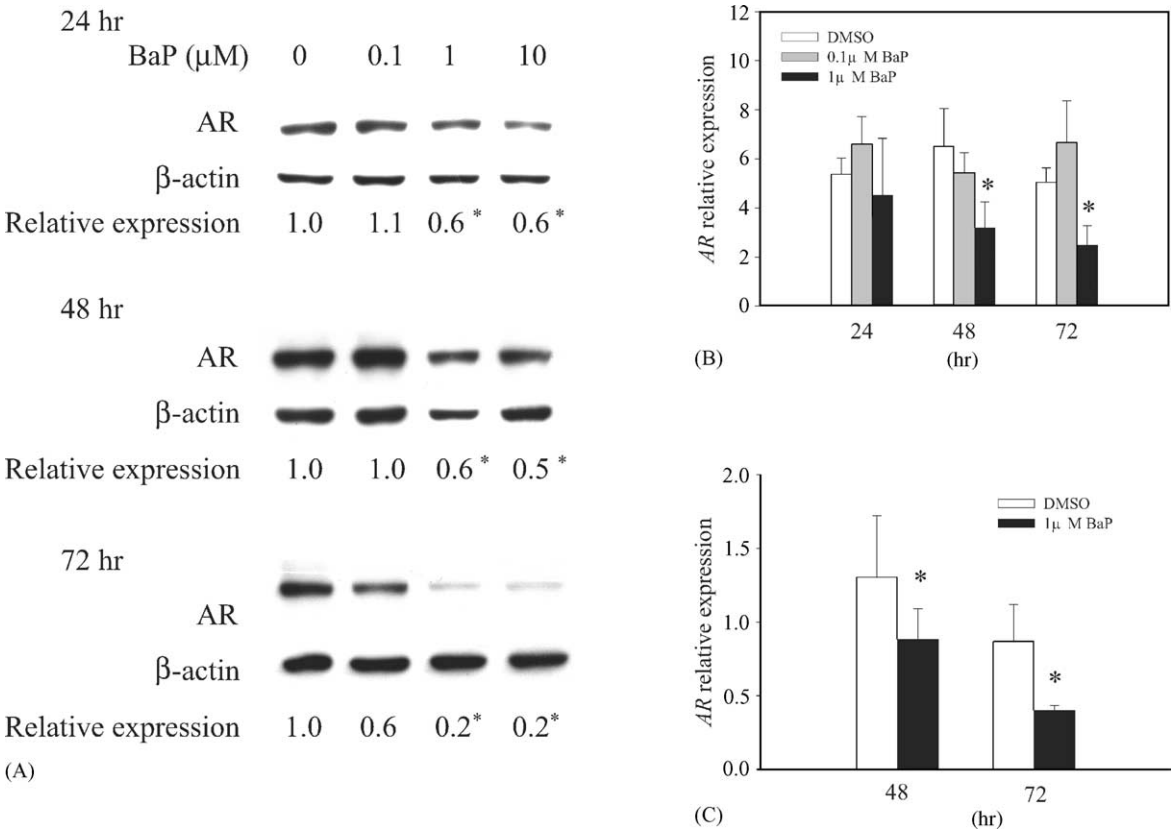

Fig. 2. Effects of BaP on AR expression in lung cells. (A) H1355 cells were treated with 0.1% DMSO, 0.1, 1 or 10 μM BaP for 24, 48 or 72 hr. AR protein was detected with Western immunoblotting. Each experiment was repeated four times. Relative expression levels were the mean of four replicates. (B) H1355 cells were treated with 0.1% DMSO, 0.1 or 1 μM BaP for 24, 48 or 72 hr and (C) BEAS-2B cells were treated with 0.1% DMSO or 1 μM BaP for 48 or 72 hr. *AR* mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with eight replicates. The symbol (*) indicates $P < 0.05$ as comparing with DMSO-treated cells.

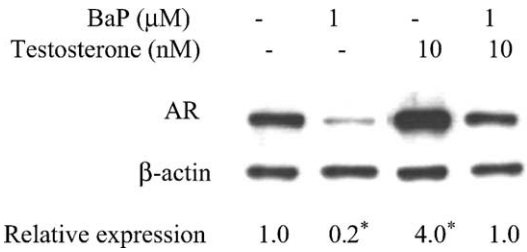

Fig. 3. Effect of BaP on testosterone-induced AR expression in H1355 cells. H1355 cells were treated with 0.1% DMSO, 1 μM BaP, 10 nM testosterone or testosterone with BaP for 72 hr. AR protein was detected with Western immunoblotting. The experiments were repeated four times. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

3.2. Effects of TCDD and PCB126 on AR expression in H1355 cells

BaP, TCDD and PCB126 are potent AhR agonists [24]. We further investigated whether TCDD and PCB126 had a similar effect on AR expression. Treatment with 1 μM BaP, 10 nM TCDD or 1 μM PCB126, respectively, increased *CYP1A1* expression levels to 100-, 1600- or 600-fold of the levels in DMSO-treated H1355 cells (Fig. 4A). Similarly, BaP, TCDD and PCB126 increased *CYP1B1* expression levels to approximately 6-fold of the levels in DMSO-treated H1355 cells (Fig. 4B). However, only BaP reduced AR expression (Fig. 4C). Neither TCDD nor PCB126 had significant effects on AR protein levels (Fig. 4C). Therefore, the induction of *CYP1A1* and *CYP1B1* by AhR agonists was not necessarily accompanied by a reduction in AR expression.

3.3. Involvement of CYP1A1/CYP1B1induction and BPDE in BaP-induced AR reduction

To understand whether BaP-induced AR reduction was dependent on *CYP1A1* or *CYP1B1* induction, H1355 cells were co-treated with BaP and AhR antagonist/cytochrome P4501 (CYP1) inhibitor (ANF) or CYP1B1-specific inhibitor (TMS) [25] for 48 hr. Treatment with either ANF, TMS alone slightly but not significantly increased AR protein levels (Fig. 5A and B). Co-treatment with 1.5 μM ANF or 5 μM TMS partially and significantly prevented the BaP-induced AR reduction in H1355 cells (Fig. 5A and B). These data suggested that AhR activation and CYP1A1/CYP1B1 activities might be required for BaP-induced AR reduction.

Since AhR activation is not sufficient to decrease AR levels, as demonstrated by TCDD and PCB126 treatment (Fig. 4), it is reasonable to speculate that BaP metabolites generated by AhR pathway might participate in BaP-induced AR reduction. When H1355 cells were directly treated with a BaP metabolite, 1 μM BPDE, for 48 hr, both AR protein and mRNA levels were significantly reduced (Fig. 5C and D).

3.4. Effects of BaP onCYP1A1, CYP1B1and AR expression in fibroblasts WI-38

Treatment with 1 μM BaP significantly induced *CYP1A1* and *CYP1B1* mRNA levels in H1355 and BEAS-2B cells, but not in WI-38 cells (Fig. 6A and B). Comparing with H1355 and BEAS-2B cells, AhR protein levels were extremely low in WI-38 cells (Fig. 6C). Con-

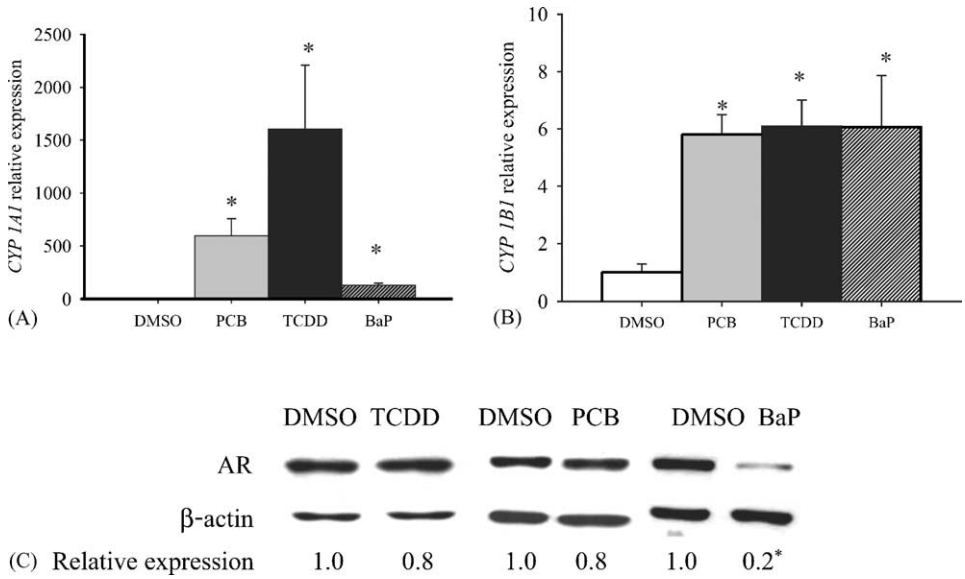

Fig. 4. Effects of BaP, TCDD and PCB126 on *CYP1A1*, *CYP1B1* and AR expression in H1355 cells. H1355 cells were treated with 0.1% DMSO, 1 μM BaP, 10 nM TCDD or 1 μM PCB126 for 24 hr. (A) *CYP1A1* and (B) *CYP1B1* mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with six replicates. (C) H1355 cells were treated with 0.1% DMSO, 1 μM BaP, 10 nM TCDD or 1 μM PCB126 for 72 hr. AR protein was detected with Western immunoblotting. Each experiment was repeated four times. Relative expression levels were the mean of four replicates. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

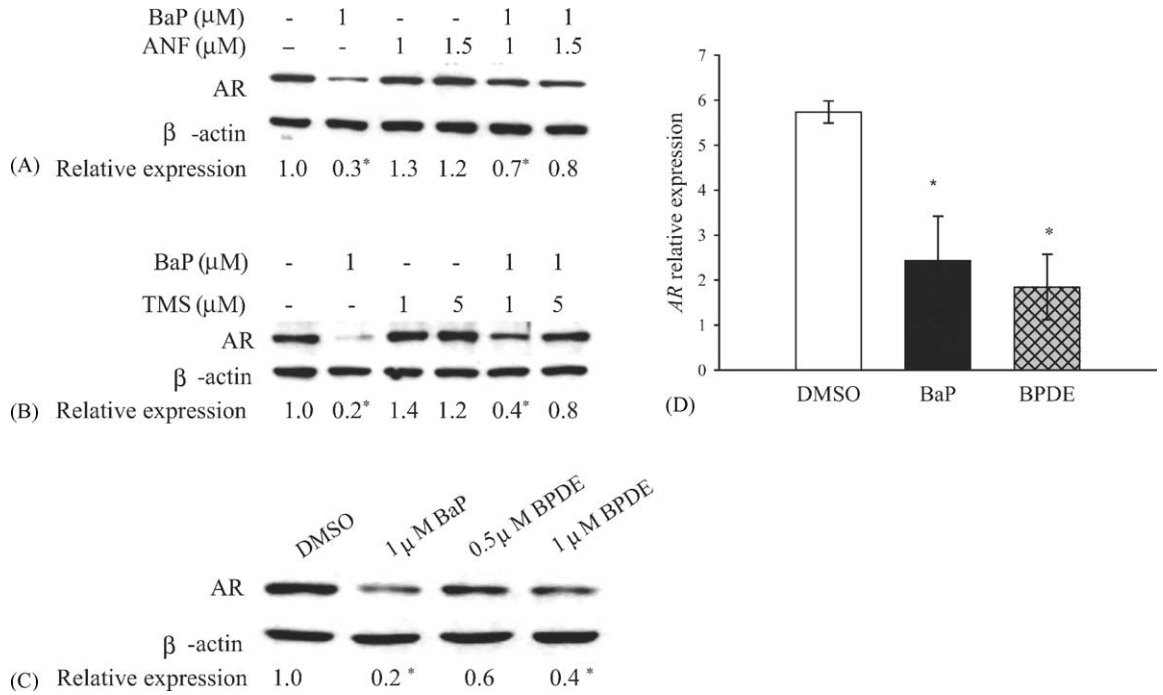

Fig. 5. Effects of BaP metabolism on BaP-induced AR reduction in H1355 cells. One micromolar BaP-treated H1355 cells were co-treated with (A) 1 or 1.5 μM ANF or (B) 1 or 5 μM TMS for 72 hr. H1355 cells were treated with 0.1% DMSO, 1 μM BaP, (C) 0.5 and 1 μM BPDE for 48 hr. AR protein was detected with Western immunoblotting. Each experiment was repeated four times. Relative expression levels were the mean of four replicates. (D) H1355 cells were treated with 0.1% DMSO, 1 μM BaP, or 1 μM BPDE for 48 hr. *AR* mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with six replicates. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

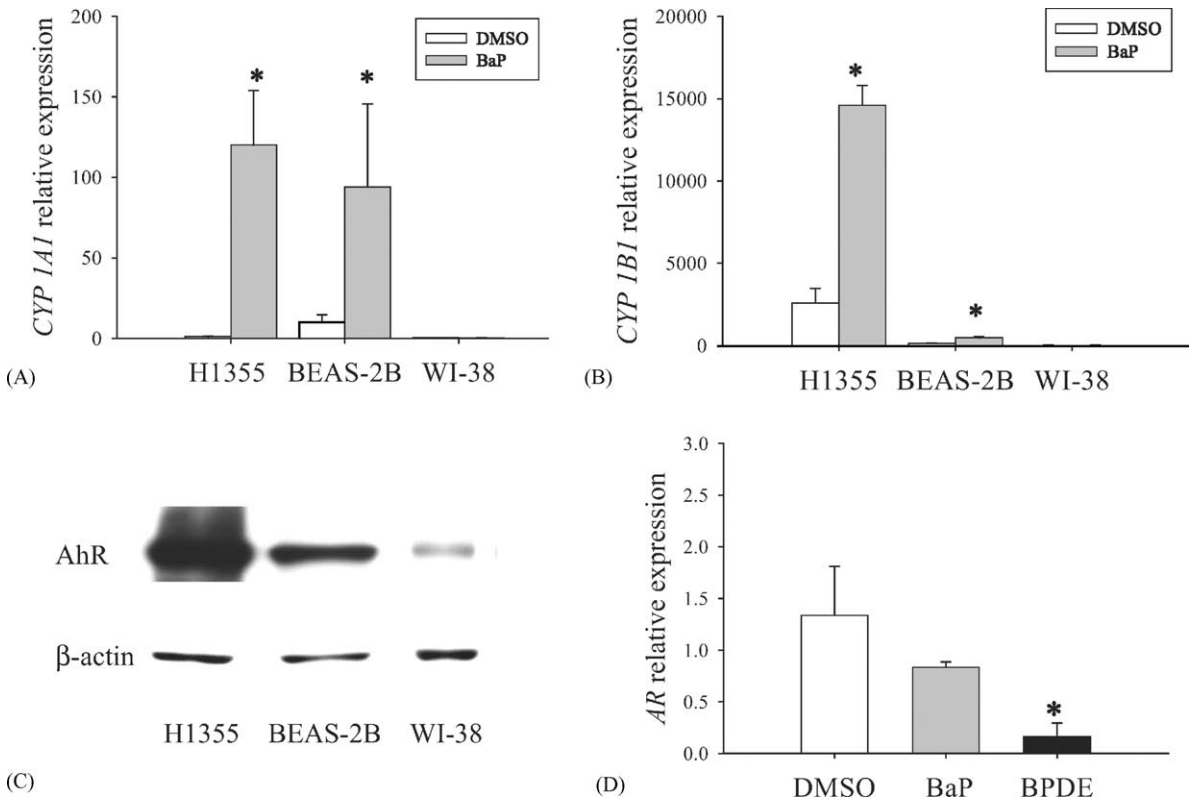

Fig. 6. Effects of BaP or BPDE on *CYP1A1*, *CYP1B1* and *AR* expression in WI-38 cells. H1355, BEAS-2B and WI-38 cells were treated with 0.1% DMSO or 1 μM BaP for 24 hr. (A) *CYP1A1* and (B) *CYP1B1* mRNA levels were quantified with the real-time RT–PCR assay. (C) AhR protein in H1355, BEAS-2B and WI-38 cells was detected with Western immunoblot. (D) WI-38 cells were treated with 0.1% DMSO, 1 μM BaP or 1 μM BPDE for 48 hr. AR mRNA levels were quantified with the real-time RT–PCR assay. Each experiment was repeated twice with six replicates. The symbol (*) indicated $P < 0.05$ as comparing with DMSO-treated cells.

sistent with the absence of *CYP1A1* and *CYP1B1* induction and low AhR expression, 1 μM BaP failed to reduce *AR* levels in WI-38 cells (Fig. 6D). Treatment with 1 μM BPDE, however, significantly reduced *AR* mRNA levels in WI-38 cells (Fig. 6D). These data demonstrated that BPDE could compensate the low induction of *CYP1A1/CYP1B1* by BaP and reduce *AR* levels in WI-38 cells.

4. Discussion

Several environmental chemicals have been reported to possess antiandrogenic properties *in vitro* and *in vivo* [26–29]. In the present study, our data represent the first observation that BaP significantly reduced AR expression in human lung cells (H1355) at both transcriptional and translational levels.

It has been reported that the AhR agonist-induced toxicity was dependent on AhR activation, which was indicated by *CYP1A1* and *CYP1B1* induction. We performed a series of experiments to investigate whether and how AhR signaling pathway would involve in BaP-induced AR reduction. First, we compared effects of different AhR agonists, TCDD, PCB126 and BaP, on AR levels. Our data showed that only BaP, but not TCDD or PCB126, reduced AR levels. It appears that either AhR signaling pathway was not involved, or AhR signaling pathway stimulated BaP metabolites production involved in AR reduction. To clarify whether AhR signaling pathway or AhR-mediated CYP1 enzyme activities play a role in BaP-induced AR reduction, BaP-treated cells were co-incubated with either AhR antagonist/CYP1 inhibitor (ANF) or CYP1B1 inhibitor (TMS). Preventive effect of ANF and TMS on BaP-induced AR reduction implied that AhR-mediated CYP1A1 and CYP1B1 activities were required for this effect. Furthermore, BaP failed to reduce AR levels in AhR non-responsive fibroblasts WI-38. These data suggested that AhR-mediated cytochrome P450 activities were specifically involved in BaP-induced AR reduction. Therefore, we proposed that BaP metabolites converted by CYP1A1 and/or CYP1B1, such as BPDE, might participate in the mechanism of AR reduction. Finally, this hypothesis was supported by our observation that BPDE directly reduced AR levels in H1355 cells and AhR non-responsive fibroblasts WI-38.

An interesting finding in our time course study was that BaP-induced AR protein reduction occurred prior to *AR* mRNA reduction. AR protein levels were slightly but significantly reduced after treatment with BaP for 24 hr. But *AR* mRNA levels were not significantly reduced until 48 hr after treatment. Furthermore, the extent of reduction in AR protein levels was greater than that in mRNA levels after 72 hr treatment. It is likely that BaP-induced AR reduction occurred through at least two different mechanisms that reflected at post-transcriptional and transcriptional levels. It has been shown that AR protein was degraded rapidly, unless bound to agonists, in cells [30]. Manin *et al.* [31] also demonstrated that DHT increased AR protein levels, but not mRNA levels in prostate cancer cells. Similar results were observed in testosterone-treated H1355 cells in our present study (data not shown). Therefore, it is possible that either BaP increased the turnover rate of AR protein or reduced the binding of androgens to AR within 24 hr. A secondary mechanism might be triggered 48 hr later, such as the conversion of BaP into BPDE, to further reduce *AR* mRNA levels.

Our present data showed that both BaP and BPDE reduced AR levels in H1355 cells. However, BaP was more effective than BPDE at the same dose (1 μM) and 1 μM BPDE was too high for a biological dose. Furthermore, ANF and TMS only partially prevented BaP-induced AR reduction. Therefore, it is likely that other mechanisms, in addition to BPDE formation, might participate in BaP-induced AR reduction. We also notice that ANF or TMS alone slightly increased AR protein levels, although the increase was not significant. In the absence of AhR agonists, CYP1A1 expression and activity was barely detectable in lung cells [9]. Nevertheless, *CYP1B1* mRNA was constitutively expressed in lung cells [9]. Treatment with ANF and TMS were expected to lower CYP1B1 expression and activity in the cells. Our data implied that a linkage between CYP1B1 activity and AR expression might exist, but more evidences are needed to establish their relationship.

BaP is shown to be converted into BPDE, which elicits genetic toxicity by forming DNA adducts and results in DNA damage [32]. Recently, BaP and BPDE were also reported to activate several signaling pathways that triggered epigenetic effects. Jyonouchi *et al.* [33] demonstrated that inhibiting PI-3 kinase activity abolished the BPDE-induced de-differentiation in airway epithelial cells. Jyonouchi *et al.* [34] also reported that BPDE increased the cytosolic calcium of airway epithelial cells. In human B cells, BPDE was shown to increase tyrosine phosphorylation [35]. Similarly, BaP was shown to increase PI-3 kinase activity, tyrosine phosphorylation or cytosolic calcium in human mammary epithelial cells and rat liver cells [36,37]. We will study the role of the PI-3 kinase, tyrosine phosphoryation and calcium in the BaP- and BPDE-induced AR reduction in the future.

BaP and BPDE reduced both the AR protein and mRNA levels in H1355 cells. From our data, it is unclear whether these chemicals decreased the rates of AR transcription or translation, or the stability of AR mRNA or protein. A few studies proposed different mechanisms for the regulation of the steady state levels of AR mRNA and protein. First, Kinoshita *et al.* [38] demonstrated that methylation of several CpG sites in the AR promoter correlated with the loss of AR gene expression in the prostate cancer cells and tissues. BPDE has been demonstrated to induce CpG methylation in mammalian cells [39], therefore, BPDE might induce the methylation of the AR promoter to

suppress AR gene transcription in H1355 cells. Second, Lin *et al.* [40] revealed that Akt mediated AR ubiquitylation and degradation. Since Akt activity was up-regulated by the PI-3 kinase signaling pathway [41], BPDE might increase AR protein degradation through the Akt signaling pathway. We will further investigate the mechanisms of AR reduction by BaP and BPDE in the future.

In utero exposure to BaP caused sterility in the male offspring of mice [42], suggesting that BaP might disturb androgen function in androgen-responsive organs. Our present study showed that H1355 cells were responsive to DHT and showed increased cell growth, nevertheless, BaP reduced AR expression in H1355 cells. Thus, BaP might interfere with lung development or surfactant production in the lungs by antagonizing AR function. Cigarette smoking and exposure to environmental tobacco smoke have been associated with higher risks of pulmonary diseases, such as lung cancer, chronic obstructive pulmonary diseases and asthma [45–47]. *In utero* passive tobacco smoke exposure was reported to affect lung function of infants [48]. BaP levels and BPDE DNA adducts were found to be significantly higher in the lung tissues of smokers than in non-smokers [43,44]. Therefore, it is plausible that BaP-induced AR reduction may interfere with AR function in the lungs and involve in the occurrence of cigarette smoking associated pulmonary diseases.

BaP was proposed to increase hormone metabolism and to disturb endocrine functions by inducing cytochrome the P4501 family. The novel finding in our present study was that BaP was metabolically activated to reduce AR expression. The reduction in AR expression may disturb androgen functions in androgen-responsive organs, such as the prostate.

Acknowledgments

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