Down-Regulation of p23 in Normal Lung Epithelial Cells Reduces Toxicities From Exposure to Benzo[a]pyrene and Cigarette Smoke Condensate via an Aryl Hydrocarbon Receptor-Dependent Mechanism

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated signaling molecule which controls tumor growth and metastasis, T cell differentiation, and liver development. Expression levels of this receptor protein is sensitive to the cellular p23 protein levels in immortalized cancer cell lines. As little as 30% reduction of the p23 cellular content can suppress the AHR function. Here we reported that down-regulation of the p23 protein content in normal, untransformed human bronchial/tracheal epithelial cells to 48% of its content also suppresses the AHR protein levels to 54% of its content. This p23-mediated suppression of AHR is responsible for the suppression of (1) the ligand-dependent induction of the *cyp1a1* gene transcription; (2) the benzo[a]pyrene- or cigarette smoke condensate-induced CYP1A1 enzyme activity, and (3) the benzo[a]pyrene and cigarette smoke condensate-mediated production of reactive oxygen species. Reduction of the p23 content does not alter expression of oxidative stress genes and production of PGE₂. Down regulation of p23 suppresses the AHR protein levels in two other untransformed cell types, namely human breast MCF-10A and mouse immune regulatory Tr1 cells. Collectively, down-regulation of p23 suppresses the AHR protein levels in normal and untransformed cells and can in principle protect our lung epithelial cells from AHR-dependent oxidative damage caused by exposure to agents from environment and cigarette smoking.

Key words: p23; AHR; aryl hydrocarbon receptor; normal cells; protein degradation.

Many cellular responses to chemical agents from our environment and diet can be mediated through the aryl hydrocarbon receptor (AHR). This receptor is a ligand-activated transcription factor which regulates a vast battery of target gene expression and cross-talks with numerous endogenous molecules to elicit diverse biological functions—effects ranging from sensing of xenobiotics (Burbach *et al.*, 1992), to modulation of immune response (Mezrich *et al.*, 2010; Nguyen *et al.*, 2010a), and control of cell growth and metastasis (Barnes-Ellerbe *et al.*, 2004; Bunaciu and Yen, 2011; Dever and Opanashuk, 2012; Fan *et al.*, 2010; Kolluri *et al.*, 2017; Silginer *et al.*, 2016). Exposure of air pollutants and cigarette smoke in the lung is known to activate pulmonary

AHR which, in turn, may affect the inflammatory status of the lung and contribute to pulmonary cell damages, leading to respiratory diseases and cancer (Rico de Souza *et al.*, 2011; Thatcher *et al.*, 2007). One of the best examples of chemicals that activate the lung AHR is benzo[a]pyrene (BaP) from cigarette smoking. BaP is an AHR ligand which up-regulates the cytochrome P450 1A1 (CYP1A1) expression in the lung (Wei *et al.*, 2002). CYP1A1 and epoxide hydrolase are well known for their involvement in bioactivation of BaP to form the carcinogenic BaP diol epoxide which alkylates DNA in the lung, leading to carcinogenesis (Bartsch, 1996). In addition, metabolism of BaP, initiated by CYP1A1, has been shown to increase reactive

oxygen species (ROS) production (Nguyen *et al.*, 2010b), which in principle may cause pulmonary oxidative stress that can lead to respiratory diseases.

The p23 co-chaperone is part of the AHR cytoplasmic complex and can affect the AHR function in many ways: p23 preserves the ligand responsiveness of AHR (Kazlauskas *et al.*, 1999); it mediates the geldanamycin-induced AHR degradation (Pollenz and Buggy, 2006); it promotes the formation of the AHR gel shift complex (Shetty *et al.*, 2003). However, the AHR levels and function were unaltered in p23-knockout embryo (Flaveny *et al.*, 2009). These p23 null mice did not survive, making it impossible to assess whether p23 is indeed dispensable for the endogenous AHR function in these mice. On the contrary, we observed that down-regulation of p23 in immortalized cancer cells—such as mouse hepatoma Hepa1c1c7, human cervical HeLa, and human hepatoma Hep3B—promotes the AHR protein degradation without ligand treatment (Nguyen *et al.*, 2012). In an effort to understand the implication of this p23 effect on AHR, we examined the effect of p23 down-regulation in normal and untransformed cells. Here we provide evidence that down-regulation of p23 causes reduction of the AHR protein levels in untransformed human lung, human breast, and mouse immune cells. Particularly, down-regulation of p23 protects human lung epithelial cells from the AHR-mediated toxicities upon exposure to BaP or cigarette smoke condensate (CSC).

MATERIALS AND METHODS

Reagents. Human bronchial/tracheal epithelial (HBTE) cells were purchased from Lifeline Cell Technology (Frederick, Maryland) and grown in BronchialLife complete medium (Lifeline Cell Technology, Frederick, Maryland). Human breast MCF-10A cells (American Type Culture Collection, Manassas, Virginia) were maintained in mammary epithelial cell growth medium (MEGM, Lonza, Walkersville, Maryland). Naïve T cells were isolated from C57BL/6 mouse spleen using the Pan T cell isolation kit from Miltenyi Biotec (Sunnyvale, California) and were subsequently differentiated into Tr1 cells in an anti-CD3 (4 ml of PBS containing 2 µg/ml of anti-CD3) coated T75 flask using TGF-β1 (2 ng/ml), IL-27 (25 ng/ml), and anti-CD28 (2 µg/ml) by incubation for 96 h at 37°C. C57BL/6 mice were purchased from Jackson Laboratory (Sacramento, California); TGF-β1 and IL-27 from R&D Systems (Minneapolis, Minnesota); anti-CD3 and anti-CD28 from eBioscience Thermo Fisher (Grand Island, New York). All cells were maintained as monolayer cultures at 37°C in an incubator with an atmosphere of 5% CO₂. BaP was purchased from Sigma (St. Louis, Missouri). Cycloheximide (CHX) was purchased from Santa Cruz Biotechnology (Santa Cruz, California). CSC was prepared from research cigarettes (3R4F; Kentucky Tobacco Research Council, Lexington, KY) on a FTC smoke machine. The total particulate matter (TPM) on the filter was calculated by the weight gain on the filter. The condensate was extracted with DMSO by soaking and rotation to prepare a 50 mg/ml solution. EcoTransfect transfection reagent was purchased from Oz Biosciences (San Diego, California). pLKO.1 p23-specific short-hairpin RNA (shRNA) plasmid (#3, CCAAATGATTCCAAGCATAAA) was purchased from Thermo Scientific (Rockford, Illinois). pLKO.1 shRNA scramble plasmids were purchased from Addgene (Cambridge, Massachusetts). The codon humanized pGFP²-N2 plasmid was purchased from BioSignal Packard (Montreal, Canada). Direct-zol RNA kit was purchased from Zymo Research (Irvine, California). MMLV high-performance reverse transcriptase was purchased from Epicentre (Madison, Wisconsin). The iQ SYBR green supermix

was purchased from Bio-Rad (Hercules, California). All human primers for *cyp1a1*, *cyp1a2*, *cyp1b1*, *nqo1*, *ugt1a1*, *ahrr*, *18s* (Table 1) were purchased from Invitrogen custom primer (Thermo Fisher Scientific, Grand Island, New York). Muse Oxidative Stress kit was purchased from EMD Millipore (Billerica, Massachusetts). Anti-AHR (SA210) polyclonal rabbit IgG was purchased from Enzo Life Sciences (Farmingdale, New York). Anti-p23 (JJ3) monoclonal mouse IgG was purchased from Thermo Fisher Scientific (Rockford, Illinois). Anti-β-actin monoclonal mouse IgG was purchased from Ambion (Austin, Texas). All secondary donkey IgGs conjugated with IRDye 800CW or 680RD were purchased from LI-COR Bioscience (Lincoln, Nebraska).

Transient transfection. For HBTE and MCF-10A cells, cells were plated at a density of 10⁵ cells per well of a 6-well plate and transfected with pLKO.1 p23-specific shRNA plasmid DNA and shRNA plasmid using EcoTransfect reagent as followed: plasmid DNA (2 µg for HBTE and 5 µg for MCF-10A cells) and EcoTransfect reagent (6 µl for HBTE and 10 µl for MCF-10A cells) were diluted into 100 µl of advanced MEM, respectively. The two solutions were mixed gently and incubated for 20 min at room temperature. Thereafter, the complexes were added into the cells growing with complete medium and incubate the cells for 72 h in a 5% CO₂ incubator at 37°C. Transfection of Tr1 cells was initiated using 4D-Nucleofector system (V4XP-3024) following the manufacturer's protocol (Lonza, Cologne, Germany). Briefly, 10⁶ Tr1 cells were resuspended into 100 µl nucleofector solution. Four microgram of plasmid DNA were added into the mixture and transferred into the transfection cuvette, then the Nucleofection process was started on the 4D-Nucleofector Core Unit. After transfection, the cells were pipetted back to the pre-warmed media and incubate the cells for 72 h in a 5% CO₂ incubator at 37°C.

Western blot analysis. Western blot analysis was performed using a previously described method (Nguyen *et al.*, 2012). Briefly, 20 µg of whole cell lysates, which were prepared in 25 mM HEPES, pH 7.4, 0.4 M sodium chloride, 1 mM EDTA, and 10% glycerol, was separated by 12% SDS-PAGE. Wet transfer was performed to transfer proteins from gel to nitrocellulose membrane at 300 mA for 120 min at 4°C. Membrane was incubated for 1 h at room temperature in blocking solution (PBS containing 5% BSA, 0.1% Tween-20, and 0.05% sodium azide), followed by primary antibody incubation with SA-210 or JJ3 overnight at 4°C. Incubation with secondary donkey antibody conjugated with IRDye was carried out in blocking solution for 2 h at room temperature. A washing step (5 times of 5 min wash with PBS + 0.1% Tween-20) was done after antibody incubation. The bands were visualized using the LI-COR Odyssey system (Lincoln, Nebraska). The intensity of AHR and p23 protein bands was quantified relative to the signals obtained for β-actin protein.

Reverse transcription-quantitative PCR. After incubation with BaP (5 µM) and CSC (5, 10, or 50 µg/ml) for 6 h, total cellular RNA was isolated from HBTE cells transfected with scramble or p23KD shRNA using Direct zol kit according to the manufacturer's instruction. The concentration of RNA was determined by measuring the absorbance at 260 nm (260/280 > 1.8) using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts). Then, the first-strand cDNA was synthesized using Epicentre MMLV reverse transcription kit. Quantitative analysis of AHR target genes expression was

Table 1. Primer Sequences Used for SYBR Green PCR

Gene	Forward Primer	Reverse Primer
<i>cyp1a1</i>	5'-GGCCACATCCGGGACATCACAGA-3'	5'-TGGGGATGGTGAAGGGGACGAA-3'
<i>cyp1a2</i>	5'-CTGGGCACTTCGACCCCTTAC-3'	5'-TCTCATCGCTACTCTCAGGGA-3'
<i>cyp1b1</i>	5'-CACCAAGGCTGAGACAGTGA-3'	5'-GATGACGACTGGGCTTACAT-3'
<i>nqo1</i>	5'-TGAAGGACCCCTGCGAACTTTC-3'	5'-GAACACTCGCTCAAACAGC-3'
<i>ugt1a1</i>	5'-TTGTCTGGCTGTTCCCACTTA-3'	5'-GGTCCGTGACATGACATCA-3'
<i>ahrr</i>	5'-GCGCCTCAGTGTGCTTACC-3'	5'-GAAGCCCAGATAGTCCACGAT-3'
18s	5'-CGCCCCCTCGATGCTCTTAG-3'	5'-CGGCGGGTCATGGGAATAAC-3'

Note: Sequences of forward and reverse primers used to amplify AHR target gene transcripts and 18S.

performed by cDNA amplification using a Bio-Rad CFX Connect real-time PCR system as described previously (Ren et al., 2016). The fold changes of gene transcript levels between treated and untreated cells were normalized by 18S. The PCR data were analyzed using $\Delta\Delta C_T$ methods (Livak and Schmittgen, 2001) by the following equation: fold change = $2^{-\Delta\Delta C_T}$, with $\Delta C_T = C_T$ (target) – C_T (18S) and $\Delta\Delta C_T = \Delta C_T$ (treated) – ΔC_T (untreated).

Ethoxyresorufin O-deethylase (EROD) assay. We performed the EROD assay to determine the CYP1A1 enzyme activity. HBTE cells were transiently transfected in 12-well plate for 66 h, then the cells were maintained with BaP (5 μ M) and CSC (5, 10, or 50 μ g/ml) for an additional 6 h. Thereafter, the cells were washed once with 0.5 ml of PBS, followed by incubation with 0.5 ml of fresh media supplemented with 2.5 μ M 7-ethoxyresorufin and 10 μ M dicumarol. After 1-h incubation in 37°C, 100 μ l of the supernatant was transferred from each well into a 96-well black plate and formation of resorufin was measured by fluorescence using a Molecular Devices Softmax spectrophotometer (excitation at 544 nm, emission at 590 nm). The relative EROD activity was calculated by subtracting the sample fluorescence with the background fluorescence in which the media contained the same ingredients in the absence of cells.

Reactive oxygen species assay. We determined the oxidative stress status of HBTE cells by quantifying the amount of superoxide present using a Muse cell analyzer according to the manufacturer's instruction (EMD Millipore, Billerica, California). Briefly, HBTE cells were transiently transfected in 12-well plate for 64 h, then CSC (5, 10, or 50 μ g/ml) and BaP (5 μ M) were added into the medium for additional 8-h incubation. Cells were then resuspended in 1 $\times$ Assay Buffer at a concentration of 10⁶/ml and stained with the dihydroethidium-based reagent for 30 min at 37°C. Intracellular ROS contents were subsequently determined.

PCR array on reactive oxygen species gene expression. Transfected HBTE cells were treated with BaP (5 μ M) in the last 6 h before harvest. RNA extraction and first-strand cDNA synthesis were performed as described under reverse transcription-quantitative PCR section. Twenty microgram of cDNA sample was mixed with 91 μ l of RNase-free water. RT² profiler PCR array was performed according to manufacturer's protocol (QIAGEN, Germantown, Maryland). Briefly, for each 96-well plate, PCR components mix were prepared by mixing 102 μ l of diluted cDNA synthesis sample with 1.350 ml of 2 $\times$ iQ SYBR Green Supermix as well as 1.248 ml of RNase-free water. Then 25 μ l of PCR components mixture was added into each well of the RT² profiler PCR array. Polymerase chain reaction was performed using following cycling conditions: hotstart at 95°C for 10 min, 40 cycles of 95°C for 15 s, followed by 60°C for 1 min.

Fluorescence readings were recorded at 60°C. The ramp rate between 95°C and 60°C was adjusted to 1°C/s. The PCR array data were analyzed using PCR array data analysis V4 based on the $\Delta\Delta C_T$ method.

PGE₂ enzyme-linked immunosorbent assay (ELISA). We used the Cayman PGE₂ ELISA kit according to the manufacturer's recommendation to measure the PGE₂ levels in HBTE cells. In brief, HBTE cells were transiently transfected in 12-well plate for 64 h with either p23-specific shRNA or scramble shRNA plasmid. After that, cells were incubated with LPS (0.05 or 5 μ g/ml) or H₂O₂ (800 μ M) for additional 8 h at 37°C. A 50 μ l aliquot of cell culture supernatant was subjected to PGE₂ measurement. The absorbance of each well was read at 412 nm using a BioTek Epoch microplate spectrophotometer.

Statistical analysis. GraphPad Prism 7 software was used to perform statistical analysis. Unpaired t-test with multiple comparisons using the Holm-Sidak method was used for Figures 1A and 2. Two-way ANOVA was used for Figure 1B whereas two-way ANOVA with Bonferroni posttest was used for Figures 3, 4B, 5A, and 5B. One-way ANOVA with Bonferroni posttest was used for Figure 4D. All data were means with error bars representing the standard deviation. The number of asterisk indicates the p-value of statistical significance as follows: $p > .05$ (ns, not significant), $p < .05$ (*), $p < .01$ (**), $p < .001$ (***), and $p < .0001$ (****).

RESULTS

Transient Transfection of the p23-Specific shRNA Reduced the p23 and AHR Protein Levels in Normal Human Lung Epithelial Cells

To address the effect of p23 on AHR expression in HBTE cells, we transiently transfected a plasmid carrying the p23-specific shRNA cDNA into cells and then measured the AHR protein levels 72 h after transfection. Transfection of the plasmid carrying the scramble shRNA cDNA was used as the negative control. We observed that both p23 and AHR were down-regulated significantly to 48% and 54%, respectively, of their positive control contents (Figure 1A). This modest p23 knockdown efficiency is expected since p23 is involved in the RISC-mediated silencing machinery and its levels should be somewhat maintained for proper cellular function (Pare et al., 2013). Transfection of a plasmid carrying a GFP cDNA was used for comparison and the results supported that knocking down of the p23 protein is responsible for the reduction of the AHR protein levels in HBTE cells. The estimation of the AHR and p23 protein contents using LI-COR Western blot analysis fell within the linear range of detection (Supplementary Figure 1).

Figure 1. Western blot analysis results showing that down-regulation of p23 reduced the AHR protein levels in HBTE cells by increasing the AHR protein degradation. β -actin was the loading control. **A**, Transient knockdown of p23 suppressed the AHR protein levels. NC, negative control, transient knockdown with a scramble shRNA plasmid; p23KD, transient knockdown of p23 using a p23-specific shRNA plasmid. The graph represents means $\pm$ SD, $n=4$. The p23 and AHR bands of pGFP transfected HBTE cells were controls that were arbitrarily set as 1 in each experiment for normalization, which were 1 ± 0.455 and 1 ± 0.422 (mean $\pm$ SD), respectively. **B**, Transient knockdown of p23 (p23KD) increased the AHR protein degradation. p23KD, transient knockdown of p23 using a p23-specific shRNA plasmid; NC, negative control, transient knockdown with a scramble shRNA plasmid. The amount of AHR protein remained after 40 μ g/ml of CHX treatment (0–6 h). The graph represents means $\pm$ SD, $n=3$. The AHR bands of NC and p23KD HBTE cells at time zero were set as 1, which were 1 ± 0.274 and 1 ± 0.239 (mean $\pm$ SD), respectively, to normalize the AHR amount remaining over time in each experiment. Images on top are a representative of the Western blot image.

Figure 2. Reverse transcription-quantitative PCR results showing that suppression of the ligand-induced AHR target gene expression in HBTE cells when p23 was down-regulated. Transcripts of 6 AHR target genes were analyzed: *cyp1a1*, *cyp1a2*, *cyp1b1*, *nqo1*, *ugt1a1*, and *ahrr*. HBTE cells, transfected with either scramble shRNA (NC) or p23-specific shRNA (p23KD), were treated with either 10 μ g/ml of CSC or 5 μ M BaP for 6 h. All graphs represent means $\pm$ SD, $n=4$.

Down-Regulation of p23 in Normal Human Lung Epithelial Cells Promoted Degradation of the AHR Protein

Next, we examined whether the reduction of the AHR protein levels in p23-knockdown HBTE cells is caused by the increase of the AHR protein degradation. After transient transfection, we

treated HBTE cells with 40 μ g/ml of CHX to inhibit general protein synthesis so that we could compare the half-life of the AHR protein of different conditions. We observed that after 6 h of CHX treatment, there were 86% and 60% of AHR remaining in the scramble shRNA-transfected and the p23-specific

Figure 3. CYP1A1 enzyme activity assay results showing that down-regulation of p23 suppressed the BaP- and CSC-induced CYP1A1 activity in HBTE cells. p23KD, transient knockdown of p23 with a p23-specific shRNA plasmid; NC, negative control, transient knockdown with a scramble shRNA plasmid. EROD (CYP1A1) activity from each experiment were normalized to represent the fold increase over control by arbitrarily set the DMSO-treated NC and p23KD HBTE cells as 1, which were 1 ± 0.405 and 1 ± 0.136 (mean $\pm$ SD), respectively. The graph represents means $\pm$ SD, $n = 3$.

shRNA-transfected HBTE cells, respectively (Figure 1B). The AHR half-life was reduced from 22 to 7.8 h when p23 was knockdown (accelerated by 2.8-fold), supporting that down-regulation of p23 in HBTE cells increases AHR protein degradation.

Down-Regulation of p23 in Normal Human Lung Epithelial Cells Suppressed the Ligand-Induced *cyp1a1* Gene Expression

To address whether knockdown of p23 would suppress AHR function, we examined whether p23-knockdown HBTE cells would exhibit reduced AHR-mediated gene transcription. We used BaP (5 µM) and CSC (10 µg/ml) to activate AHR and measured the amount of six AHR target gene transcripts—namely *cyp1a1*, *cyp1a2*, *cyp1b1*, *nqo1*, *ugt1a1*, and *ahrr*—in HBTE cells transfected with either a scrambled shRNA or p23-specific shRNA plasmid. We observed that only *cyp1a1* gene transcription was consistently compromised when the p23 content was down-regulated (Figure 2). The CSC-mediated *ahrr* gene transcription was also reduced when p23 was knockdown; however, the BaP-mediated *ahrr* gene transcription was not altered. Although appreciable amount of *cyp1b1* gene transcription was measured when HBTE cells were treated with either BaP or CSC, the extent of this gene transcription was not affected by the p23 content. We cannot rule out the possibility that BaP and CSC affect *cyp1b1* gene transcription via an AHR-independent mechanism. The remaining three AHR target genes—namely *cyp1a2*, *nqo1*, and *ugt1a1*—were induced to a much lesser extent in HBTE cells and not affected by the p23 content. It is conceivable that expression of AHR target genes is cell-specific and many of them are not induced via AHR in HBTE cells. Collectively, *cyp1a1* was the most AHR responsive gene in HBTE cells and its induction was affected by the p23 contents.

Down-Regulation of p23 in Normal Human Lung Epithelial Cells Suppressed the Benzo[a]pyrene- and Cigarette Smoke Condensate-Induced CYP1A1 Enzyme Activity

To assess whether reduction of the AHR content to 54% in HBTE cells, as a result of p23 knockdown, would negatively affect the

normal AHR function, we performed the EROD assay to determine the ligand-induced CYP1A1 activity. We used BaP, which is a known AHR ligand, and CSC, which contains BaP and other ingredients that might be AHR ligands, to activate the AHR-dependent *cyp1a1* gene expression. We observed that the EROD activities were all significantly suppressed in the p23-knockdown HBTE cells when compared with the HBTE cells transfected with a scramble shRNA plasmid (Figure 3). Specifically, down-regulation of p23 in HBTE cells suppressed the CSC-induced (5–50 µg/ml) EROD activity to 32–45% and the BaP-induced (5 µM) activity to 49% when compared with the values in HBTE cells with normal p23 content. It is very evident from this EROD experiment that 46% reduction of the AHR content in HBTE cells definitely has functional implication.

Down-Regulation of p23 in Normal Human Lung Epithelial Cells Suppressed the Benzo[a]pyrene- or Cigarette Smoke Condensate-Mediated Reactive Oxygen Species Production

Next, we examined whether down-regulation of p23 in HBTE cells alters the ROS production caused by BaP or CSC. In addition to BaP, other CSC ingredients can cause ROS production. We treated the HBTE cells transfected with either a scramble shRNA or p23-specific shRNA plasmid with BaP (5 µM) or CSC (5–50 µg/ml) for 8 h and then measured the ROS levels using a Muse cell analyzer. We observed that 5 µM BaP generated ROS in about 34% of the total cell population in the scramble shRNA transfected cells and this amount of ROS production was significantly reduced to 14% when p23 was down-regulated (Figs. 4A and 4B). Similar trend was observed with the 10 µg/ml of CSC treatment—reduction of ROS production was observed from 35% to 19%, which was equaled to suppression of ROS production to 54% when p23 was down-regulated. However, higher (50 µg/ml) or lower (5 µg/ml) concentration of CSC did not show any difference in ROS production, suggesting that lower than 10 µg/ml of CSC might not be sufficient to trigger ROS production and higher than 10 µg/ml of CSC generated ROS via a p23 independent mechanism. When we repeated the same experiment using Jurkat cells which lack CYP1A1 activity, we observed background amount of ROS formation in all conditions except 50 µg/ml of CSC, suggesting that CSC at high concentration produced ROS via a CYP1A1 independent mechanism (Figs. 4C and 4D). Hydrogen peroxide was used as the positive control to confirm that ROS production was feasible in Jurkat cells.

Treatment of Benzo[a]pyrene Did Not Alter Oxidative Gene Expression in Normal Human Lung Epithelial Cells When p23 Was Down-Regulated

Next, we examined whether suppression of BaP-mediated ROS production by reduced p23 content in HBTE cells (Figure 4B) could be mediated by changes in oxidative stress gene expression. We obtained cDNA from the BaP-treated scramble shRNA-transfected and p23-knockdown HBTE cells. Knockdown of the p23 content in these cells was confirmed by LI-COR Western blot analysis before we proceeded with the experiment. Results from the PCR array study showed that there was no significant changes in 84 oxidative stress genes that were analyzed (Table 2). Five genes—*epx*, *prex1*, *gpx5*, *gpx6*, and *ttn*—showed >2-fold changes; however, the C_T numbers were all beyond 30 which made them unreliable.

Figure 4. Cell analysis results showing that down-regulation of p23 suppressed the BaP- or CSC-mediated ROS production in HBTE cells. Jurkat cells were used as a control for CYP1A1 independent ROS production. Histograms represent the amount of normal (blue/dark shade, M1) and ROS-producing (red/light shade, M2) HBTE cells (A) and Jurkat (C) upon CSC (5–50 µg/ml) or BaP (5 µM) treatment. About 800 µM H₂O₂ treatment is the positive control to make sure that ROS can be produced in Jurkat cells. DMSO was the negative control. p23KD, transient knockdown of p23 with a p23-specific shRNA plasmid; NC, negative control, transient knockdown with a scramble shRNA plasmid. Graph (B) represents summary of HBTE cells (A) $n = 5$ whereas graph (D) represents summary of Jurkat (C) $n = 3$. Both graphs represent means $\pm$ SD.

Down-Regulation of p23 Reduced the AHR Protein Levels in Untransformed Human MCF-10A and Normal Mouse Tr1 Cells

Next, we examined whether down-regulation of p23 in other normal cells could reduce AHR protein levels. We performed the transient knockdown experiment using either untransformed human breast MCF-10A cells or mouse Tr1 cells. We observed that the p23 and AHR protein levels were significantly reduced to 62% and 79%, respectively, when the p23-knockdown MCF-10A cells were compared with the scramble shRNA transfected control cells (Figure 5A). For controls, we transfected the cells with either a GFP expressing plasmid or a scramble shRNA expressing plasmid and observed that the p23 and AHR protein levels were not significantly altered. Expression of AHR in naïve

T and Tr1 cells showed that the differentiation was successful since we expected minimal AHR expression in naïve T cells but robust AHR expression in Tr1 cells. As we compared the p23-knockdown and the scramble shRNA-transfected mouse Tr1 cells, we observed that when the p23 content was down-regulated to 58% content, the AHR protein levels were significantly reduced to 57% content (Figure 5B).

Knockdown of p23 Did Not Affect the PGE₂ Levels in Human Lung Epithelial Cells

Next, we investigated whether reduced p23 content would reduce the PGE₂ levels in HBTE cells since p23 has been shown to

Table 2. Oxidative Stress Gene Expression Analysis Results Comparing BaP-Treated Scramble shRNA Transfected HBTE Cells (Control Sample) With BaP-Treated p23-Knockdown HBTE Cells (Test Sample)

Gene Name	Fold Up- or Down-Regulation (C_q)	Gene Name	Fold Up- or Down-Regulation (C_q)	Gene Name	Fold Up- or Down-Regulation (C_q)
ALB	1.34 (25.1)	GSTP1	1.18 (18.09)	PRDX6	1.15 (21.82)
ALOX12	1.00 (29.01)	GSTZ1	1.22 (26.47)	PREX1	9.06 (34.5)
AOX1	1.02 (25.81)	GTF2I	1.09 (21.38)	PRNP	-1.01 (20.25)
APOE	1.01 (24.64)	HMOX1	1.26 (26.46)	PTGS1	-1.07 (28.04)
ATOX1	1.19 (22.49)	HSPA1A	1.05 (22.07)	PTGS2	-1.23 (23.31)
BNIP3	-1.02 (21.89)	KRT1	-1.18 (32.58)	PXDN	-1.17 (22.38)
CAT	1.14 (23.59)	LPO	-1.93 (31.03)	RNF7	1.18 (23.02)
CCL5	1.06 (25.01)	MB	-1.32 (30.3)	SCARA3	-1.56 (28.68)
CCS	1.40 (26.57)	MBL2	-1.71 (32.38)	VIMP	1.17 (23.53)
CYBB	-2.13 (31.46)	MGST3	1.06 (22.4)	SEPP1	-1.01 (26.83)
CYGB	-1.03 (20.72)	MPO	-1.66 (32.95)	SFTPD	-1.22 (29.14)
DHCR24	1.07 (22)	MPV17	1.17 (24.15)	SIRT2	1.15 (24.31)
DUOX1	1.10 (23.97)	MSRA	-1.03 (26.52)	SOD1	1.14 (22.09)
DUOX2	1.21 (26.04)	MT3	1.07 (30.39)	SOD2	1.17 (21.01)
DUSP1	1.68 (23.51)	NCF1	1.55 (19.03)	SOD3	-1.32 (27.79)
EPHX2	1.06 (27.72)	NCF2	1.05 (22.76)	SQSTM1	1.05 (18.74)
EPX	22.23 (38.52)	NOS2	-1.32 (28.19)	SRXN1	1.04 (22.67)
FOXO1	-1.13 (28.04)	NOX4	1.01 (29.22)	STK25	-1.01 (23.24)
FTH1	1.17 (15.9)	NOX5	1.09 (29.23)	TPO	-1.12 (30.16)
GCLC	1.19 (22.4)	NQO1	1.47 (22.25)	TTN	-2.62 (31.57)
GCLM	1.40 (23.86)	NUDT1	-1.31 (27.28)	TXN	1.22 (20.38)
GPX1	1.21 (19.15)	OXR1	1.06 (23.81)	TXNRD1	1.32 (21.7)
GPX2	1.28 (22.18)	OXSRI	-1.05 (23.74)	TXNRD2	-1.01 (26.37)
GPX3	1.03 (22.33)	PDLIM1	1.08 (20.52)	UCP2	1.46 (24.71)
GPX4	1.15 (20.56)	PNKP	1.05 (24.61)	ACTB	-1.05 (16.55)
GPX5	-2.26 (32.45)	PRDX1	1.13 (24.01)	B2M	1.00 (17.27)
GPX6	-4.13 (30.63)	PRDX2	1.10 (22.06)	GAPDH	-1.06 (17.22)
GPX7	-1.01 (27.51)	PRDX3	1.09 (20.79)	HPRT1	1.09 (25.45)
GSR	-1.41 (25.06)	PRDX4	1.03 (21.84)	RPLP0	1.02 (18.46)
GSS	1.73 (23.67)	PRDX5	1.24 (21.3)		

Notes: Cells were treated with 5 μ M BaP for 6 h. The “-” symbol indicates downregulation. C_q is the threshold cycle of control samples. When the C_q is relatively high (> 30), it indicates that the expression level of the gene is very low and unreliable.

be a PGE₂ synthase. We confirmed that the p23 levels were indeed transiently knockdown before we proceeded with the ELISA analysis (Figure 6, top). Results from ELISA showed no significant change of PGE₂ levels when p23 was knockdown (Figure 6, bottom). Although there were some reduction of PGE₂ levels when HBTE cells were treated with H₂O₂ or LPS, these changes were not observed when p23 was down-regulated. Collectively, changes of the PGE₂ levels were not consistent with the PGE₂ synthesis activity of p23.

DISCUSSION

Down-regulation of p23 expression in liver and cervical cancer cell lines causes degradation of the AHR protein, leading to suppression of the AHR function (Nguyen *et al.*, 2012). This effect of p23 on AHR is not Hsp90-dependent since p23 mutants with minimal Hsp90 interaction can restore the AHR protein levels in p23-knockdown HeLa cells (Pappas *et al.*, 2018). Although ablation of p23 is fatal, experiments using the p23 null mouse embryos revealed that p23 does not alter the AHR expression and function (Flaveny *et al.* 2009). One may argue that the p23-dependent AHR degradation occurs only in immortalized cells. Here we have shown that the AHR expression in normal, untransformed cells (as in the mouse embryos) is susceptible to p23 control since down-regulation of p23 in three untransformed cells, namely human lung HBTE, human breast MCF-

10A, and mouse immune Tr1, consistently shows reduction of the AHR protein levels. We suspected that there is some yet undefined compensatory mechanisms *in vivo* which cause p23 to be dispensable for AHR function in mouse embryos.

When p23 is transiently down-regulated in HBTE cells, the AHR protein levels can be reduced to 54% of the wild-type levels due to increased AHR protein degradation. This seemingly insufficient reduction has definite downstream functional implications: the AHR-induced, CYP1A1-dependent EROD activity is significantly reduced and the amount of ROS production triggered by BaP or CSC is also suppressed. We did not observe any apparent cytotoxicity when we treated the HBTE cells with 5 μ M BaP (Supplementary Figure 2). On the contrary, CSC was cytotoxic particularly at the highest dose we used to conduct our experiments: the cell viability dropped to 85%, 62%, and 37% when HBTE cells were treated with 5, 10, and 50 μ g/ml of CSC, respectively (Supplementary Figure 2). We observed that BaP causes ROS production in cell lines in a manner that is AHR-dependent (Nguyen *et al.*, 2010b), consistent with the observation that ROS can be generated from BaP quinone metabolites which are generated downstream to the AHR-dependent CYP1A1 metabolism of BaP (Penning *et al.*, 1999). Our data are consistent with the finding in human pulmonary mucocarcinoma cell line NCI-H292 that 1 μ M BaP caused ROS production which could be blocked by an AHR antagonist resveratrol (Chiba *et al.*, 2011). These researchers showed that

Figure 5. Western blot analysis results showing that down-regulation of p23 suppressed the AHR protein levels in MCF-10A (A) and mouse Tr1 (B) cells. β-actin was the loading control. (A) WT, wild-type untreated; pGFP, transient transfection of a GFP expressing plasmid (pGFP²-N2); NC, negative control, transient knockdown with a scramble shRNA plasmid; p23KD, transient knockdown of p23 with a p23-specific shRNA plasmid. β-actin was the loading control. This graph represents means ± SD, n = 4. The p23 and AHR bands of WT were controls that were arbitrarily set as 1, which were 1 ± 0.222 and 1 ± 0.237 (mean ± SD), respectively, to normalize all values for each experiment as fold over control. (B) naïve T, NT, undifferentiated T cells; Tr1, Type 1 regulatory T cells; NC, transient negative control knockdown Tr1; p23KD, transient p23 knockdown Tr1. This graph represents means ± SD, n = 4 except naïve T, NT (n = 3). The p23 and AHR bands of Tr1 were controls that were arbitrarily set as 1, which were 1 ± 0.317 and 1 ± 0.364 (mean ± SD), respectively, to normalize all values for each experiment as fold over control.

Figure 6. ELISA results showing the PGE₂ levels in HBTE cells. HBTE cells transfected with either p23-specific shRNA (p23KD) or scramble shRNA negative control (NC), were treated with vehicle (water), LPS (0.05 or 5 μg/ml final concentration) or H₂O₂ (800 μM final concentration). Top images are a representative of replicate data. Bottom graph represents means ± SD, n = 3.

up-regulation of the AHR-dependent mucin 5AC expression by BaP was caused by ROS, since antioxidant (such as N-acetyl-cysteine) could abolish this up-regulation. It was observed that “factors” in the *ahr* null mouse lung increased the AHR-mediated reporter luciferase response, suggesting the presence of endogenous ligands that are more abundant when AHR is absent (Chiaro et al., 2007). This observation is consistent with the

hypothesis that there is endogenous AHR ligand which is susceptible for CYP enzyme degradation. However, AHR may have opposing effect on ROS production: it has been reported that AHR lowers the risk of developing hyperoxic injury in fetal mice, which may be linked to less oxygen-mediated ROS production in lung endothelial cells (Zhang et al., 2015). Differences in the AHR-related ROS production are likely cell-specific—AHR in lung epithelial cells may respond differently than in lung endothelial cells and fibroblasts. Nevertheless, since the epithelial cells are the frontline defense in the lung against harmful agents from inhalation, it is important to know that targeting p23 may limit damages from AHR-mediated ROS production.

The *ahr* null mice are more prone to lung inflammation (Thatcher et al., 2007). Lung fibroblasts derived from *ahr* null mice showed significantly more inflammation when treated with CSC (Baghole et al., 2008), which might be related to the ROS levels in response to CSC treatment. One explanation is that AHR may stabilize RelB—without AHR, RelB is less and in turn can cause inflammatory responses. Increased inflammation with CSC treatment in AHR^{-/-} fibroblasts may partly due to increased COX-2 and PGE₂ levels. It is known that the COX-2 dependent synthesis of PGE₂ is mediated through microsomal GST-like I protein (Murakami et al., 2000). In contrast, p23 catalyzes PGE₂ synthesis downstream of the COX-1 action (Tanioka et al., 2000). Nonetheless, we explored the possibility that down-regulation of p23 might reduce PGE₂ content, affecting the inflammatory status of HBTE cells. It is interesting to note that when AHR function was suppressed by knocking down the p23 content, PGE₂ levels were essentially unchanged, suggesting that p23 does not contribute to the production of PGE₂ in HBTE cells. We were able to measure the PGE₂ levels in HBTE cells, which was similar to what was reported in human lung A549 cells using the same PGE₂ kit (Gabasa et al., 2013), suggesting

that PGE₂ may participate in the inflammatory process of HBTE cells in a p23-independent manner. Furthermore, the p23-deficient embryo produced 50% less of PGE₂ levels; however, primary fibroblast isolated from these p23-deficient mice showed 2.5-fold higher in PGE₂ levels, suggesting that mechanisms other than p23 serving as a PGE₂ synthase defines the final PGE₂ content (Lovgren *et al.*, 2007). Surprisingly, treatment of H₂O₂ (800 μM) and LPS (5 μg/ml) caused less PGE₂ production (instead of more) in scramble shRNA-transfected HBTE cells. These effects, however, were not observed in p23-knockdown HBTE cells. Our treatment of HBTE cells with H₂O₂ and LPS clearly did not trigger PGE₂ production. Reduction of p23 content in HBTE cells caused more, but not less, PGE₂ synthesis, suggesting that the function of p23 as a PGE₂ synthase was not observed in HBTE cells. Collectively, we concluded that knockdown of p23 causes less AHR function, leading to reduced ROS production upon BaP and CSC treatment; this effect is not related to the function of p23 as a PGE₂ synthase.

Interestingly, the reduction of the CSC-mediated ROS production in p23-knockdown HBTE cells was only observed at 10 μg/ml dose; higher CSC dose showed similar ROS production in untreated and p23-knockdown HBTE cells. We believe that some ingredients in CSC promote ROS production in a CYP1A1 independent manner at a higher dose because only higher CSC dose caused increased in ROS production in Jurkat cells which lack CYP1A1 function (Nguyen *et al.*, 2010b). Thus, down-regulation of p23 has a protective effect on the CSC-mediated ROS production which is partly AHR dependent.

Down-regulation of p23 should potentially affect the AHR function in the lung by reducing its protein content. Kynurenine, an endogenous AHR ligand, has been implicated for lung inflammation. Expression of kynurenine can be induced by toll-like receptor ligands in mouse lung, leading to reduction of lung inflammation and airway hyperreactivity (Hayashi *et al.*, 2004). IDO, an enzyme upstream to the biosynthesis of kynurenine, can be expressed in lung epithelial cells and its expression leads to inhibition of inflammatory response, partly via reduction of the IL-1β-activated IL-6 expression (Lee *et al.*, 2017). On the contrary, IL-24, an AHR target gene, can be up-regulated in human bronchial epithelial cell line BEAS-2B, which may in turn lead to inflammation (Luo *et al.*, 2017). It would be of interest to utilize p23 knockdown cells to explore the complex role of AHR in lung inflammation.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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