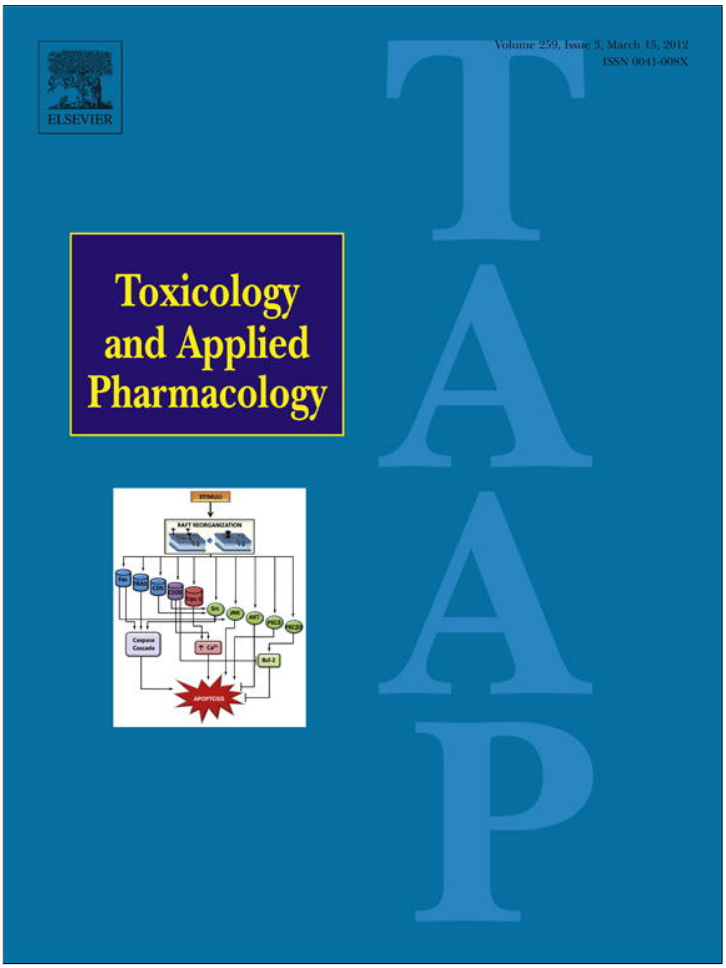

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Aryl hydrocarbon receptor protects lung adenocarcinoma cells against cigarette sidestream smoke particulates-induced oxidative stress

Ya-Hsin Cheng [a], Su-Chin Huang [b], Chun-Ju Lin [b], Li-Chuan Cheng [b], Lih-Ann Li [b,*]

[a] Graduate Institute of Basic Medical Science, School of Medicine, China Medical University, Taichung 40402, Taiwan, ROC
[b] Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Zhunan, Miaoli 35053, Taiwan, ROC

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ABSTRACT

Environmental cigarette smoke has been suggested to promote lung adenocarcinoma progression through aryl hydrocarbon receptor (AhR)-signaled metabolism. However, whether AhR facilitates metabolic activation or detoxification in exposed adenocarcinoma cells remains ambiguous. To address this question, we have modified the expression level of AhR in two human lung adenocarcinoma cell lines and examined their response to an extract of cigarette sidestream smoke particulates (CSSP). We found that overexpression of AhR in the CL1-5 cell line reduced CSSP-induced ROS production and oxidative DNA damage, whereas knockdown of AhR expression increased ROS level in CSSP-exposed H1355 cells. Oxidative stress sensor Nrf2 and its target gene NQO1 were insensitive to AhR expression level and CSSP treatment in human lung adenocarcinoma cells. In contrast, induction of AhR expression concurrently increased mRNA expression of xenobiotic-metabolizing genes CYP1B1, UGT1A8, and UGT1A10 in a ligand-independent manner. It appeared that AhR accelerated xenobiotic clearing and diminished associated oxidative stress by coordinate regulation of a set of phase I and II metabolizing genes. However, the AhR-signaled protection could not shield cells from constant oxidative stress. Prolonged exposure to high concentrations of CSSP induced G0/G1 cell cycle arrest via the p53–p21–Rb1 signaling pathway. Despite no effect on DNA repair rate, AhR facilitated the recovery of cells from growth arrest when CSSP exposure ended. AhR-overexpressing lung adenocarcinoma cells exhibited an increased anchorage-dependent and independent proliferation when recovery from exposure. In summary, our data demonstrated that AhR protected lung adenocarcinoma cells against CSSP-induced oxidative stress and promoted post-exposure clonogenicity.

Introduction

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor. Xenobiotics such as dioxins and polycyclic aromatic hydrocarbons (PAHs) can bind to AhR as ligands and activate its transcriptional activity. Activated AhR induces expression of cytochrome P450 enzymes, thus initiating the metabolism and clearing of xenobiotics. However, if the oxygenated metabolites generated by cytochrome P450 enzymes are not rapidly metabolized by phase II enzymes, these oxygenated metabolites have a chance to attack macromolecules and cause oxidative stress (Shimada, 2006). In addition, activated oxygen may dissociate with cytochrome P450 in the form of superoxide radical before completion of the oxygenation reaction (Zangar et al., 2004). Therefore, AhR plays a dual role in xenobiotic metabolism, detoxification and activation of xenobiotics.

Increasing evidence suggests that AhR-signaled metabolism is involved in carcinogenesis and tumor transformation of lung cancer. Studies on AhR polymorphisms in Chinese and Korean populations revealed that AhR haplotypes influenced the risk of lung cancer (Chen et al., 2009; Kim et al., 2007). Lin et al. (2003) observed that AhR expression was higher in lung adenocarcinoma tumor cells than adjacent normal bronchiolar epithelial cells (Lin et al., 2003). High AhR expression was coupled with increased expression of cytochrome P450 monooxygenase 1B1 (CYP1B1) in early-stage lung adenocarcinoma specimens regardless of smoking status (J.T. Chang et al., 2007). Oyama et al. (2007) also observed that AhR expression was more frequent in early-stage or highly differentiated lung adenocarcinoma than other non-small cell lung cancers. AhR expression in adenocarcinoma was positively correlated to expression of cytochrome P450 genes CYP1A1, CYP2E1 and CYP3A (Oyama et al., 2007).

Adenocarcinoma is the major histological type of lung cancer found in nonsmokers. Exposure to environmental cigarette smoke is known for decades to increase the risk of lung adenocarcinoma in nonsmokers (IARC, 2004; Morabia and Wynder, 1991; Perng et al., 1996). Environmental cigarette smoke comprises about 85% sidestream smoke emitted from smoldering cigarettes between puffs

* Corresponding author at: Division of Environmental Health and Occupational Medicine, National Health Research Institutes, R1-5034, 35 Keyan Rd., Zhunan, Miaoli 35053, Taiwan, ROC. Fax: +886 37 587406.
E-mail address: lihann@nhri.org.tw (L.-A. Li).

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and 15% mainstream smoke exhaled by smokers (IARC, 2004). Cigarettes produce significantly more PAHs and aromatic amines in sidestream smoke than in mainstream smoke, and most PAHs are present in the particulate phase of cigarette smoke. Tar content and filter have no effects on the composition of PAHs in sidestream smoke. Cigarette sidestream smoke particulates (CSSP) appear to be a major source of PAHs in indoor ambient air (Grimmer et al., 1987; Lodovici et al., 2004). The high expression frequencies of AhR and CYP enzymes in lung adenocarcinoma lead to a hypothesis that environmental cigarette smoke promotes the progression of lung adenocarcinoma though AhR-regulated metabolism.

However, the question of whether AhR-regulated metabolism intensifies cigarette smoke-induced oxidative stress or confers anti-oxidant protection to tumor cells by accelerating detoxification remains unclear. To address this question, we investigated the impact of AhR expression alteration on cellular response to CSSP exposure in human lung adenocarcinoma CL1-5 and H1355 cells. Results showed that elevation of AhR expression reduced the production of reactive oxygen species (ROS) and attenuated oxidative DNA damage in CSSP-treated cells. On the contrary, suppression of AhR expression increased ROS production. AhR-overexpressing lung adenocarcinoma cells exhibited increased clonogenicity when recovery from exposure.

Materials and methods

CSSP preparation and treatment. Cigarette smoke was generated from a Taiwanese brand named Long Life Cigarette (white package) following an international standard procedure. Sidestream smoke particulates were captured onto glass fiber filters and extracted with methanol as described in a previous study (Lee et al., 2011). Dried particulates were dissolved in DMSO and stored in aliquots at −80 °C. The particulates were added to phenol red-free RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) in 1000-fold dilution. 3% charcoal/dextran-treated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) was included in medium during treatment except comet assays.

Construction of CL1-5(TO-AhR). AhR cDNA was cloned into a tetracycline-regulated expression vector pTO/DsRed in which the Tet-on expression unit was derived from pcDNA4/TO/myc-HisA (Invitrogen) and the selection markers geneticin resistance and red fluorescent protein were from pDsRed-N1 (Clontech Laboratories, Mountain View, CA, USA). The resulting plasmid pTO-AhR/RFP was cotransfected with a blasticidin-resistant tetracycline repressor expression plasmid pcDNA6/TR (Invitrogen) at a 1:10 ratio into the human lung adenocarcinoma cell line CL1-5 (Yang et al., 1992). Transformants was selected with 1 mg/ml geneticin (Sigma-Aldrich) and 10 μg/ml blasticidin (Sigma-Aldrich). AhR expression could be induced by addition of 1 μg/ml doxycycline (Dox; Invitrogen).

Reporter transfection. The transactivation activities of AhR and Nrf2 in CL1-5(TO-AhR) were assessed by transient transfection of cognate reporter plasmids into cells using Lipofectamine 2000 (Invitrogen). The reporter plasmids 4xDRE-TATA-Luc and CYP1B1-Luc were used to test AhR activity, while 2xARE-SV40-Luc was used to examine Nrf2 activity. SV40-βgal was cotransfected as an internal control. Dox (1 μg/ml) was given to the transfected cells 16 h before a 24-h treatment with either 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Supelco, Bellefonte, PA, USA) or CSSP at indicated concentrations. The luciferase activity activated by treatment was measured and normalized to β-galactosidase activity as described previously (Lin et al., 2006).

Western blot. Nuclear and cytoplasmic protein extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL, USA). Whole-cell protein extraction and Western blot were performed as described previously (Tsou et al., 2005). Primary antibodies used in this study are rabbit anti-AhR (BML-SA210) from Enzo Life Sciences International (Plymouth Meeting, PA, USA), rabbit anti-Nrf2 (sc-722) and mouse anti-β-actin (sc-47778) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-phosphor-p53(Ser15) (#9284), mouse anti-p21 (#2946), mouse anti-Rb1 (#9309), rabbit anti-phospho-Rb1(Ser780) (#9307), and rabbit anti-histone H3 (#9715) from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated goat anti-rabbit IgG from Santa Cruz Biotechnology and goat anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) were used to recognize the corresponding primary antibodies.

Alkaline comet assay. Oxidative base lesions caused by treatment were determined by alkaline comet assays (Cheng et al., 2007). Briefly, cells were embedded in the middle low-melting gel of a sandwich agarose gel coated on a coverslip. Cells were lysed by overnight incubation in 10 mM Tris, pH 10, 2.5 M NaCl, 100 mM EDTA, 1% N-laurylsarcosine, 1% Triton X-100, and 10% DMSO at 4 °C followed by neutralization in 10 mM Tris–HCl (pH7.4) for 10 min. DNA was digested with 0.5 unit of endonuclease III (Trevigen, Gaithersburg, MD, USA) followed by 1 unit of formamidopyrimidine-DNA glycosylase (Trevigen) at 37 °C for 1 h each enzyme before being unwound in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH13.4) for 20 min. Electrophoresis was conducted at 25 V/300 mA for 25 min. DNA was then neutralized with 0.4 M Tris–HCl (pH7.5) and stained with 4 μg/ml propidium iodide (Sigma-Aldrich). The images of 50 cells per coverslip were captured by a fluorescence microscope equipped with a CoolSNAP CCD camera. The extent of DNA damage was measured in tail moment using software CometScore (Olive et al., 1990).

ROS determination. Intracellular ROS level in response to treatment was determined by measuring the conversion of fluorescent dichlorofluorescin (DCFH) from DCFH diacetate (DCFH-DA) through ROS-mediated oxidation (Bass et al., 1983). 10 μM DCFH-DA (Eastman Kodak, Rochester, NY, USA) was added to cells 30 min before treatment ended. DCFH fluorescence was measured using Coulter Epics XL-MCL™ Flow Cytometer (Beckman Coulter, Miami, FL, USA) with excitation at 485 nm and emission at 545 nm.

Real-time RT-PCR and RT-PCR array. Real-time RT-PCR was performed as described in a previous study (Lin et al., 2011). Gene expression levels were determined by calibration against standard curves and normalized to β-actin expression levels. RT-PCR array analysis was conducted following the array user manual. Total RNA isolated using Roche High Pure RNA Isolation kit (Basel, Switzerland) was reverse transcribed using SABioscience RT^2 first strand kit and real-time PCR amplified on SABiosciences Cell Cycle PCR Array (Frederick, MD, USA) in an ABI Prism 7900 HT instrument equipped with ABI Prism 7900 SDS software 2.1 (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the RT^2 Profiler PCR array data analysis template downloaded from the SuperArray Web site (http://www.sabioscience.com).

Cell viability assay. The density of viable cells was determined by measuring the absorbance of formazan metabolites converted from tetrazolium salt WST-8 (Dojindo Laboratories, Kumamoto, Japan) as recommended by the manufacturers.

Cell cycle analysis. Cells were stained with 20 μg/ml propidium iodide, 0.1% Triton-X, and 0.2 mg/ml RNase A (Sigma-Aldrich) in PBS at 37 °C for 30 min after overnight fixation in 70% ethanol at −20 °C. The percentages of 10,000 cells in the G0/G1, S, and G2/M stages in each sample were determined using Coulter Epics XL-MCL™ Flow Cytometer (Beckman Coulter) and software WinMDI 2.8 (http://facs.scripps.edu/software.html).

Colony formation assay. Cells were suspended in 0.5% low-melting agarose (Sigma-Aldrich) and laid over 0.6% agarose (Amresco, Solon, OH, USA) in 6-well plates at 1000/well. Agarose was dissolved in 10% serum-containing medium. 1 μg/ml Dox was included in top agarose and liquid medium to induce AhR expression in cells. Colonies were stained with 0.05% crystal violet (Sigma-Aldrich) after 5 weeks of cultivation, and those with a diameter no less than 0.5 mm were counted using Image J (http://rsb.info.nih.gov.ij).

Statistics. All data are expressed as means ± SE. Dose effects were analyzed by one-way ANOVA coupled with Turkey's HSD post hoc test, whereas pair comparisons were performed using independent-samples *t*-test (SPSS, Chicago, IL, USA). In addition, ANOVA and general linear model (SPSS) were employed to analyze the effects of AhR induction and CSSP pretreatment on colony formation.

Results

CSSP activated AhR Signaling dose-dependently in an AhR-inducible cell line CL1-5(TO-AhR)

To study the role of AhR in lung adenocarcinoma, an inducible AhR-expressing cell line was established from the human lung adenocarcinoma CL1-5 cell line by stable transfection with a tetracycline-regulated AhR expression system. Western blot confirmed that AhR protein was undetectable in the whole-cell extract of CL1-5(TO-AhR) until 1 μg/ml Dox (tetracycline analogue) was given to the transgenic cell line. The induction elicited by Dox was specific to AhR because β-actin protein levels remained constant regardless of Dox treatment (Fig. 1A). Transfection of CL1-5(TO-AhR) cells with an AhR responsive reporter 4xDRE-TATA-Luc showed that Dox treatment (1 μg/ml, 24 h) raised reporter activity by about 9-fold in the absence of exogenous ligand. Addition of the prototype ligand TCDD (10 nM) together with Dox further increased reporter activity to 16.5-fold as compared to the uninduced vehicle control (Fig. 1B). Data demonstrated that the recombinant AhR protein induced by Dox was function intact and acted in both exogenous ligand-dependent and independent manners.

A large amount of CSSP extract was prepared from a popular local brand of cigarettes and tested on CL1-5(TO-AhR). The extract showed a positive dose–response effect on the 1.1-kb promoter of CYP1B1, a well-known AhR target gene in lung cells. Induction of AhR overexpression in CL1-5(TO-AhR) by Dox treatment (1 μg/ml, 24 h) simultaneously increased expression of the Luc reporter gene from the 1.1-kb CYP1B1 promoter of CYP1B1-Luc. A 24-h treatment with CSSP significantly increased the reporter activity of CYP1B1-Luc at 6 μg/ml in the absence of Dox and at 0.6 μg/ml in the presence of Dox. The increase raised by 60 μg/ml CSSP was 4.36 ± 0.23-fold in AhR-uninduced cells and 26.55 ± 0.04-fold in AhR-induced cells (Fig. 1C). AhR expression level in cells influenced the strength of CSSP to induce the AhR gene battery.

AhR overexpression attenuated CSSP-induced oxidative DNA damage in lung adenocarcinoma cells

We further examined the impact of AhR overexpression on CSSP-induced oxidative stress. Base lesions caused by oxidative stress was determined by coupling an alkaline comet assay with strand excision enzymes that recognized oxidized bases specifically (Wang et al., 2002). While undamaged DNA retained a compact core structure, DNA containing oxidative base lesions was fragmented by excision enzymes and electrophorized away from the core in reverse proportion to fragment size. The magnitude of oxidative DNA damage was measured in DNA tail moment. Although a 30-min treatment with 60 μg/ml CSSP produced a less amount of oxidative base lesions than 400 μM H_2O_2 (positive control), CSSP caused more severe DNA damage than vehicle. CSSP-caused base lesions increased with the time of treatment. Maximal DNA damage appeared within 1–2 h of treatment (Fig. 2A). Induction of AhR expression 16 h before a 1.5-h treatment with 60 μg/ml CSSP significantly reduced oxidative DNA damage (Fig. 2B). The decrease was not due to an increase in DNA repair. Base lesions in Dox-treated and untreated CL1-5(TO-AhR) cells decreased in parallel to a steady state within a 3-h recovery course after removal of CSSP, suggesting that DNA damage was repaired in similar rates regardless of AhR expression level (Fig. 2C).

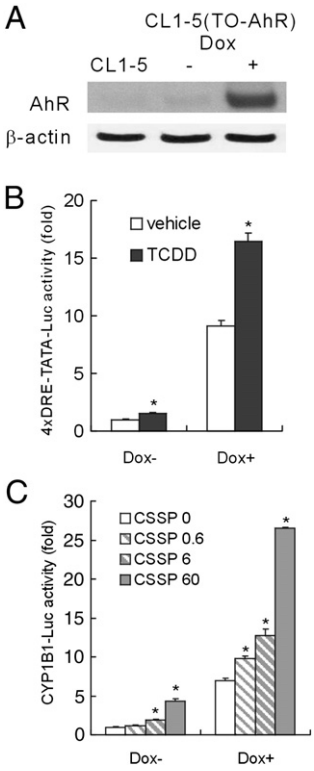


Fig. 1. Tet-on induction of AhR expression and activity in CL1-5(TO-AhR) cells. (A) Western blot analysis confirmed that AhR protein expression was induced in CL1-5(TO-AhR) after a 24-h treatment with 1 μg/ml Dox. The parental CL1-5 cell line was used as a negative control, whereas β-actin protein was as a house-keeping control. (B) AhR activity in the presence and absence of Dox-induced AhR expression was measured by transfection of CL1-5(TO-AhR) cells with the AhR responsive reporter 4xDRE-TATA-Luc. AhR activity was highly induced by Dox and positively responsive to a 24-h treatment with 10 nM TCDD ($n=6$). (C) The dose response of AhR activity to CSSP treatment was determined by CYP1B1-Luc transfection. 0–60 μg/ml CSSP was given to the transfected cells alone or together with Dox for 24 h ($n=3$). * $p<0.005$ as compared to the vehicle control.

AhR expression level influenced CSSP-induced ROS production in lung adenocarcinoma cells

We speculated that CSSP caused less oxidative DNA damage in AhR-overexpressing cells because of a lower production of ROS in response to exposure. To verify this speculation, we examined ROS levels in both Dox-treated and untreated CL1-5(TO-AhR) cells using a sensitive fluorogenic assay. CL1-5(TO-AhR) cells exhibited higher fluorescence emission when treated with 60 μg/ml CSSP for 1.5 h compared to vehicle treatment. Induction of AhR expression 16 h before CSSP treatment significantly reduced fluorescence emission, indicating that high levels of AhR expression lowered CSSP-induced ROS production in lung adenocarcinoma cells (Fig. 3).

To verify the influence of AhR on CSSP-induced ROS production, we also examined the ROS response in the human lung adenocarcinoma H1355 cell line and its AhR knockdown derivatives (si1414-6 and si1414-7) in which AhR expression was stably reduced by short interfering RNA (J.T. Chang et al., 2007). Western blot confirmed the

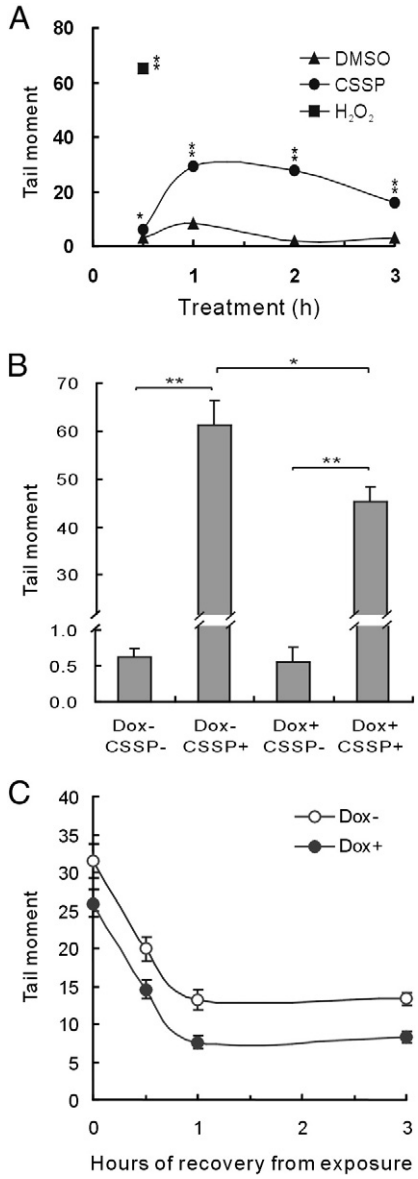


Fig. 2. AhR overexpression attenuated CSSP-caused oxidative DNA damage but had no effect on DNA repair rate. (A) Course of oxidative DNA damage under CSSP exposure. CL1-5(TO-AhR) cells were challenged with 60 μg/ml CSSP for 0.5–3 h in serum-free medium. 0.1% DMSO and 400 μM H_2O_2 were used as vehicle and positive controls, respectively. Oxidative DNA lesions were determined by alkaline comet assay. Damage levels were presented as tail moment. (B) The impact of AhR overexpression on CSSP-caused DNA damage was assayed by induction of AhR expression with 1 μg/ml Dox 16 h before a 1.5-h treatment with vehicle or 60 μg/ml CSSP. (C) DNA damage level was monitored for 3 h after termination of the 1.5-h challenge. The magnitude of DNA damage decreased in comparable rates to the plateau phase in Dox-treated and untreated cells, suggesting that AhR overexpression had little effect on DNA repair. Each experiment was repeated at least twice and the representative one was shown. * $p<0.05$ and ** $p<0.005$.

reduction of AhR protein expression in the AhR knockdown derivatives (Fig. 4A). Exposure of H1355 and its derivatives to CSSP (60 μg/ml, 1.5 h) increased ROS-mediated fluorescence emission (Figs. 4B and C). The intracellular ROS level was significantly higher in the AhR knockdown derivatives (Figs. 4D and E). These results prove that AhR expression level determines ROS production in lung adenocarcinoma cells. High AhR expression decreases CSSP-induced ROS production.

Effects of AhR overexpression on Nrf2 and metabolizing genes

Coordination of phase I and II metabolizing gene expression is crucial in defense against xenobiotic-induced oxidative stress. The reactive metabolites produced during phase I metabolism need conjugative modification by phase II metabolizing enzymes before excretion from cells. Many phase II metabolizing genes are under the regulation of a redox-sensitive transcription factor Nrf2. AhR has been suggested to regulate phase II gene expression by activating Nrf2 signaling (Kohle and Bock, 2007). Therefore, we examined the impact of AhR induction on Nrf2 protein expression by Western blot and Nrf2 transactivation activity by reporter transfection in this study. While expression of AhR protein was significantly induced in CL1-5(TO-AhR) cells by Dox treatment, Nrf2 protein abundance remained steady in the cytosol and nucleosol. Neither Dox nor CSSP (60 μg/ml, 1.5 h) caused perceptible changes in Nrf2 protein level (Fig. 5A). Treating AhR-induced and uninduced CL1-5(TO-AhR) cells with various doses of CSSP for 1.5 h also did not change the activity of the Nrf2 responsive reporter 2xARE-SV40-Luc (Fig. 5B). The Nrf2-dependent reporter activity remained comparable between AhR-induced and uninduced cells when the 60 μg/ml CSSP treatment was lengthened to 6 h (Fig. 5C). These results indicated that (1) Nrf2 was not responsible for the lower ROS production detected in Dox/CSSP-treated CL1-5(TO-AhR) cells; and (2) AhR had no direct or immediate effect on Nrf2 activity in human lung adenocarcinoma cells.

We further examined expression levels of metabolizing genes in Dox-treated and untreated CL1-5(TO-AhR) cells by RT-PCR analysis. The AhR target gene CYP1B1 was highly responsive to Dox-elicited AhR induction. However, treating either AhR-induced or uninduced cells with 60 μg/ml CSSP for 1.5 h did not improve CYP1B1 mRNA expression as compared with vehicle treatment (Fig. 6A). UDP-glucuronosyltransferases (UGTs) catalyzing glucuronidation of endogenous and exogenous compounds are the major enzymes responsible for the elimination of CYP-generated reactive metabolites. A recent study demonstrates that AhR and Nrf2 collaboratively regulate transcription of the human UGT1A8 and UGT1A10 genes through adjacent binding sites in the promoter (Kalthoff et al., 2010). Our analysis showed that mRNA expression of both UGT genes was significantly increased in AhR-overexpressing cells. In contrast to CYP11B1, UGT1A8 and UGT1A10 mRNA levels were elevated by CSSP treatment although the upregulation in AhR-overexpressing cells lacked statistical significance (Figs. 6B and C). In rodent cells, AhR is indispensable for TCDD to induce NAD(P)H:quinone oxidoreductase 1 (NQO1), the best-characterized Nrf2-regulated antioxidant gene (Favreau and Pickett, 1991; Ma et al., 2004). However, our data showed that AhR overexpression and CSSP treatment had no effect on NQO1 mRNA expression in human CL1-5(TO-AhR) cells (Fig. 6D). The insensitivity of NQO1 to AhR in CL1-5(TO-AhR) seemed explicable. In contrast to the rat NQO1 gene, no functional AhR binding motif has been identified in the human NQO1 promoter (Favreau and Pickett, 1991; Radjendirane and Jaiswal, 1999). In addition, little difference in NQO1 expression between CSSP-treated and untreated cells suggested that a short term of exposure to CSSP did not produce ROS as much as necessary to trigger Nrf2-regulated antioxidant defense.

Effects of AhR overexpression on cell viability, cell cycle progression, and post-exposure clonogenicity of CSSP-treated lung adenocarcinoma cells

We also examined the effect of constant CSSP exposure on the viability of CL1-5(TO-AhR) in the presence and absence of Dox. As compared to vehicle control, constant exposure to 60 μg/ml CSSP impaired the viability of both AhR-induced and uninduced CL1-5 (TO-AhR) cells, and the harm in cell viability increased with exposure time. Induction of AhR overexpression significantly improved cell viability when CSSP treatment only lasted 24 h. About 87% of AhR-induced CL1-5(TO-AhR) cells survived from the 24-h CSSP treatment in contrast to 69% of AhR-uninduced cells. However, the protective effect stemming from AhR overexpression gradually diminished when CSSP treatment was lengthened. The difference in viability between

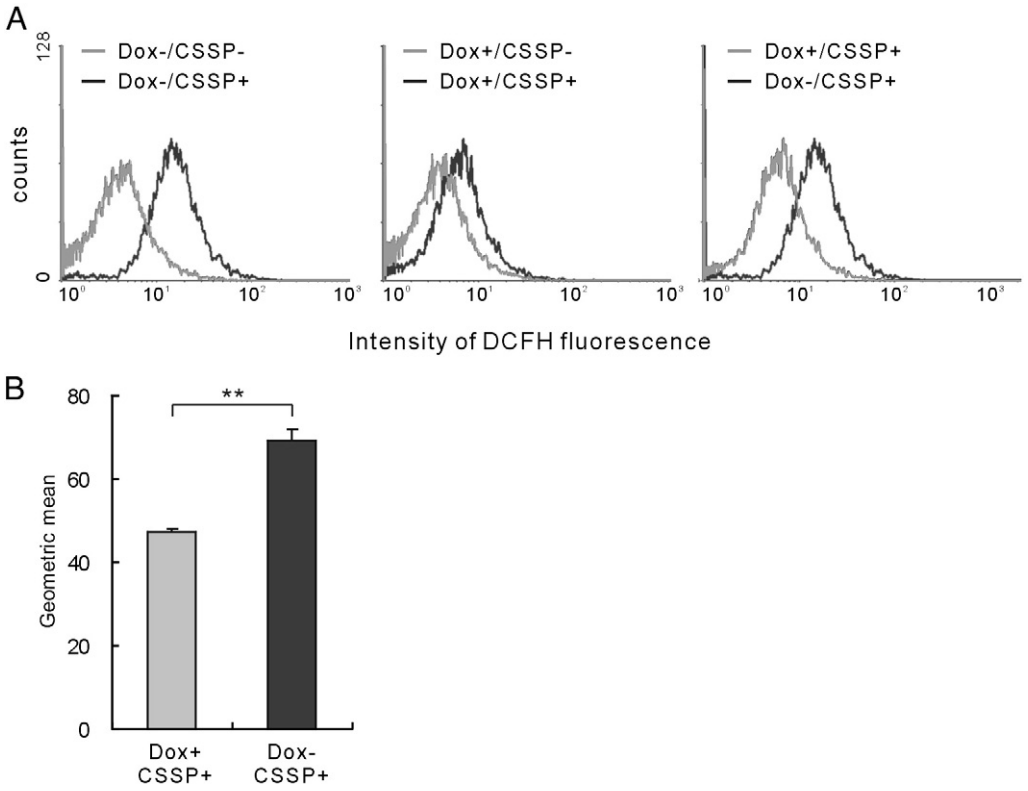


Fig. 3. AhR overexpression reduced CSSP-induced ROS production in CL1-5(TO-AhR). (A) Intracellular ROS level was determined by ROS-mediated DCFH fluorescence emission. A 1.5-h treatment with 60 μg/ml CSSP (CSSP+) resulted in higher fluorescence emission than vehicle treatment (CSSP-). Induction of AhR expression by 1 μg/ml Dox (Dox+) 16 h before the 1.5-h treatment reduced CSSP-induced fluorescence emission. (B) Geometrical means of fluorescence emission in CSSP-treated cells in the presence and absence of Dox induction ($n=4$). $^{**}p<0.005$.

AhR-induced and uninduced cells was extinguished after 72 h of exposure (Fig. 7A).

Flow cytometry analysis indicated that cells regardless of AhR expression level were arrested in the G0/G1 phase after 72 h of treatment with 60 μg/ml CSSP. The percentage of G0/G1 phase was increased by 25–27% to the detriment of the S and G2/M phases. However, CSSP had no effect on cell cycle distribution when the dose was reduced to 20 μg per ml or below (Fig. 7B). Examination of cell cycle gene expression using a RT-PCR array revealed that the 72-h treatment with 60 μg/ml CSSP upregulated mRNA expression of cyclin-dependent kinase inhibitor (CDKI) p21 2.29-fold ($p=0.0006$) but downregulated CDK substrate Rb1 1.63-fold ($p=0.0025$). Expression of CDKI p27 remained unchanged, whereas the mRNA abundances of CDKI p15 and p16 were too low to be detected (Fig. 7C). Western blot analysis confirmed that protein expression of p21 and Rb1 varied consistently with mRNA expression. Although the abundance of Rb1 protein was decreased in CSSP-treated cells, most of Rb1 protein was present in the nonphosphorylated form that blocked the entry of cell cycle into the S phase (Fig. 7D) (Sun et al., 2007). Although the CSSP treatment only slightly increased p53 mRNA level 1.36-fold ($p=0.0311$) (Fig. 7C), the treatment considerably increased phosphorylation of p53 protein (Fig. 7D). Collectively, these results suggested that prolonged exposure of AhR-overexpressing cells to high concentrations of CSSP could cause sufficient stress to induce G0/G1 cell cycle arrest via the p53–p21–Rb1 signaling pathway.

Although exposure of CL1-5(TO-AhR) cells to 60 μg/ml CSSP led to AhR-independent growth arrest, we observed that cells resumed growth once free from exposure. When equal numbers of CL1-5(TO-AhR) cells were seeded into CSSP-free medium after 72 h of pretreatment with vehicle or 60 μg/ml CSSP, CSSP-pretreated cells grew faster than vehicle-pretreated cells. Induction of AhR overexpression by Dox during pretreatment and recovery enhanced anchorage-dependent proliferation, particularly proliferation of CSSP-pretreated cells (Fig. 8A). Furthermore, AhR-overexpressing cells exhibited an increased capability to form colonies when recovery from exposure. In this experiment, cells were incubated in CSSP-free agarose medium after 72 h of pretreatment. Colonies with a minimum diameter of 0.5 mm were counted after 5-week cultivation. AhR had little effect on anchorage-independent proliferation of vehicle-pretreated cells. Similar numbers of colonies were formed from vehicle-pretreated cells despite the presence and absence of Dox. CSSP pretreatment increased colony formation. ANOVA test showed there was a significant difference between groups ($p=0.006$). General linear model analysis further indicated a positive relationship between CSSP pretreatment and colony number ($p=0.002$). However, the CSSP-promoted increase lacked *t*-test significance in the absence of AhR induction. Upon AhR expression was induced by Dox, CL1-5(TO-AhR) cells formed a significantly higher number of colonies when recovery from CSSP pretreatment than recovery from vehicle pretreatment (40.0 ± 2.0 vs. 25.2 ± 2.5 colonies) (Fig. 8B). High AhR expression appeared to facilitate CSSP-promoted clonogenicity.

Discussion

High levels of AhR were frequently detected in lung adenocarcinoma, the most prevalent form of lung cancer found in never smokers (Lin et al., 2003; Oyama et al., 2007). Environmental cigarette smoke is an important lung cancer risk factor for never smokers (IARC, 2004). In this study, we examined the impact of AhR expression level on environmental cigarette smoke-induced oxidative stress in lung adenocarcinoma cells. Exposure of lung adenocarcinoma cells to high concentrations of CSSP, the major component of environment cigarette smoke, caused DNA damage alongside ROS elevation. CYP-catalyzed metabolism had been regarded as a key mechanism by which tobacco PAHs increased cellular ROS formation and caused genotoxicity (Kitamura and Kasai, 2007). Increased expression of AhR was assumed to enhance cigarette smoke-associated damage by upregulation of CYP gene expression.

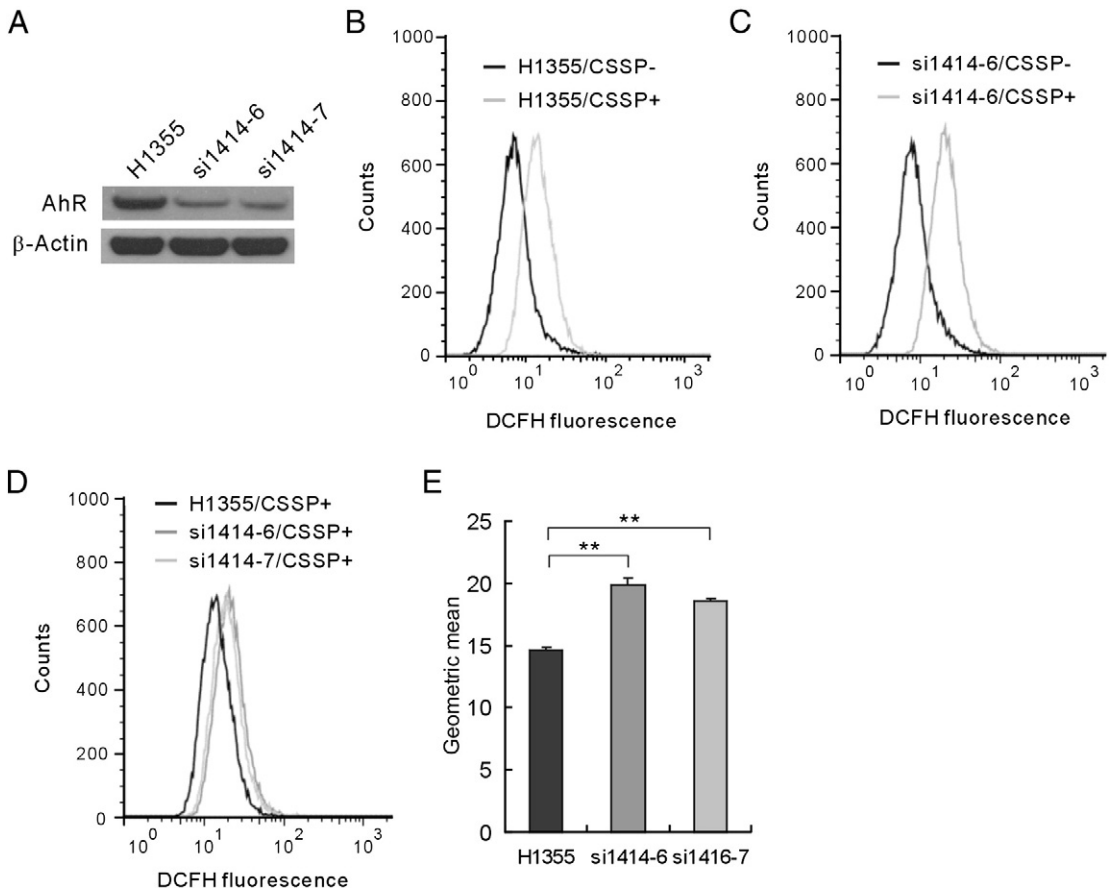


Fig. 4. Knockdown of AhR expression increased CSSP-induced ROS production. (A) Western blot analysis confirmed that AhR protein expression was reduced in two H1355 derivative cell lines, si1414-6 and si1414-7 that stably expressed AhR siRNA. (B–C) Treating H1355 and si1414-6 cells with 60 μg/ml CSSP for 1.5 h (CSSP+) gave rise to higher ROS-mediated DCFH fluorescence emission than vehicle treatment (CSSP−). (D) The 1.5-h CSSP treatment induced higher fluorescence emission in the AhR-knockdown si1414-6 and si1414-7 cell lines as compared to the parental H1355 cell line. (E) Geometrical means of fluorescence emission in CSSP-treated cells ($n=4$). $^{**}p<0.005$.

However, our data showed that AhR overexpression reduced ROS level and DNA damage in CSSP-treated lung adenocarcinoma cells. On the other hand, knockdown of AhR expression increased exposure-induced ROS production. It appeared that AhR favored metabolic clearance over metabolic activation in lung adenocarcinoma cells under the conditions of this study.

Nrf2 regulating transcription of a battery of antioxidant genes is an important regulator in protection against oxidative stress. AhR has been suggested to interact with Nrf2 in three ways: (1) direct regulation of Nrf2 gene expression, (2) indirect activation of Nrf2 signaling via CYP-generated metabolites, and (3) functional interaction in the promoter region of antioxidant genes (Kohle and Bock, 2007).

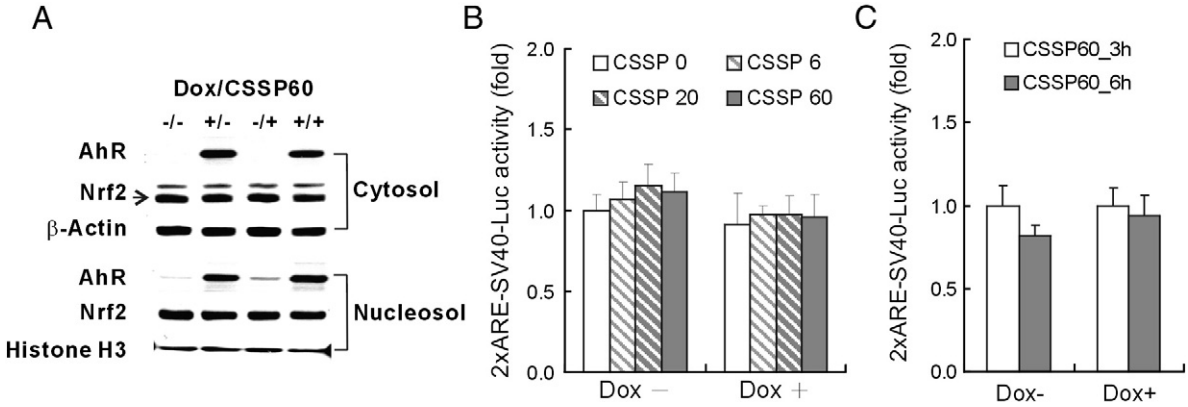


Fig. 5. AhR overexpression had no effect on Nrf2 expression and activity in CL1-5(TO-AhR). (A) CL1-5(TO-AhR) cells were treated with or without Dox (1 μg/ml) overnight. Vehicle or CSSP (60 μg/ml) was then added for an additional 1.5-h treatment before cytosol and nucleosol fractionation. Western blot analysis showed that AhR protein was induced and translocated into the nucleus under the Dox treatment, whereas Nrf2 protein level remained constant in the cytosol and nucleosol regardless of treatment. The protein levels of β-actin and histone H3 were also examined as cytoplasmic and nuclear house-keeping controls, respectively. (B–C) The transactivation activity of Nrf2 in response to various CSSP treatments in the presence and absence of AhR induction was assayed by transfection of the Nrf2-responsive 2xARE-SV40-Luc reporter into CL1-5(TO-AhR). The transfected cells were given 0–60 μg/ml CSSP for 1.5 h (B) or 60 μg/ml CSSP for 3 and 6 h (C) after an overnight incubation with or without 1 μg/ml Dox. There were no significant differences between different treatments ($n=4$).

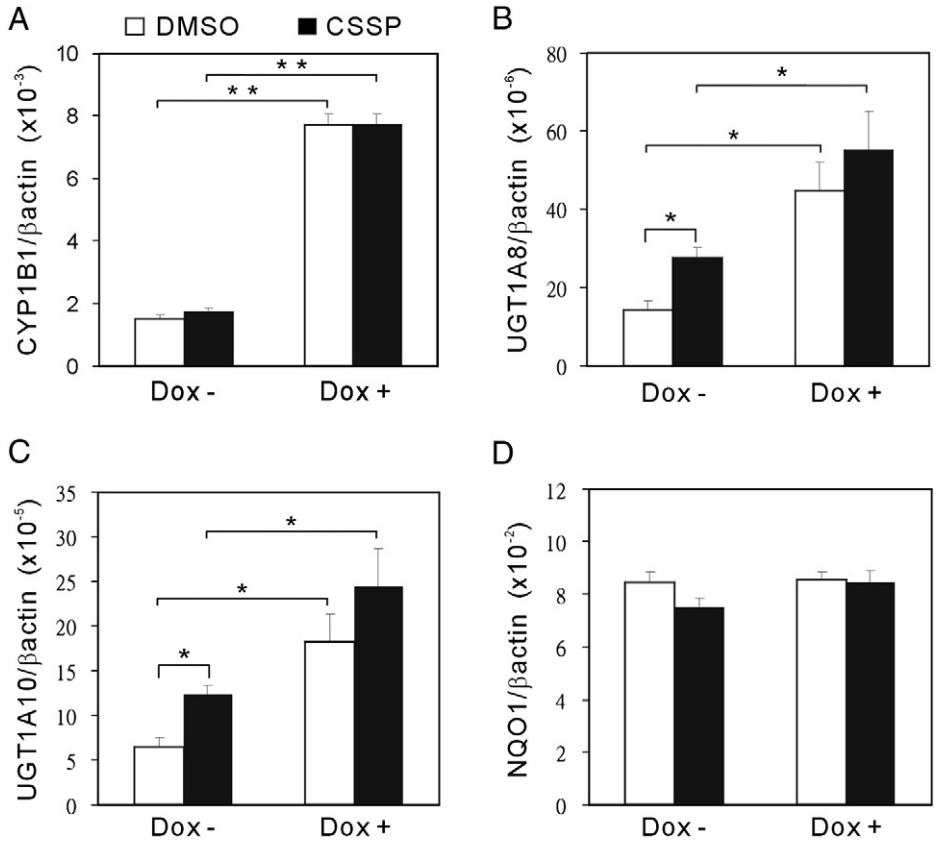
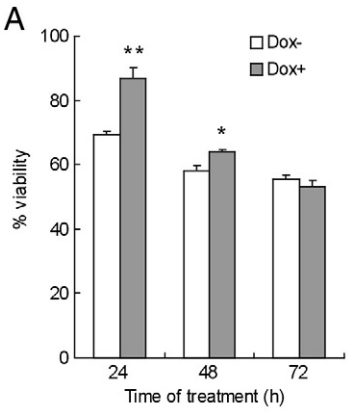


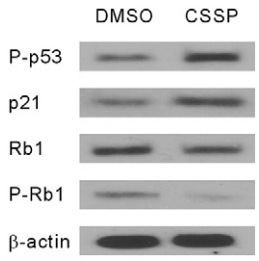
Fig. 6. Expression of metabolizing genes in CL1-5(TO-AhR) under various Dox/CSSP treatments. CL1-5(TO-AhR) cells were treated with or without 1 μg/ml Dox overnight before addition of 60 μg/ml CSSP or 0.1% DMSO (vehicle) for an additional 1.5-h treatment. Total RNA was isolated after treatment for real-time RT-PCR analysis. The transcript abundances of CYP1B1 (A), UGT1A8 (B), UGT1A10 (C), and NQO1 (D) were normalized to that of β-actin. $^{*}p<0.05$ and $^{**}p<0.005$.



C

	Fold Up-or Down-Regulation	T-TEST p value
p53	1.36	0.0311
p21	2.29	0.0006
p27	1.00	0.9963
p15	nd	----
p16	nd	----
Rb1	-1.63	0.0025

nd: below detection limit



B

Cell cycle distribution (%) of CL1-5(TO-AhR) after a 72-h treatment

CSSP (μg/ml)	Dox −			Dox +		
	G0/G1	S	G2/M	G0/G1	S	G2/M
0	38.50±0.52	31.51±0.50	29.99±0.74	36.74±0.91	33.21±1.13	30.05±0.70
2	37.50±0.58	32.35±0.84	30.15±0.65	38.67±0.80	30.64±0.32	30.69±0.94
6	38.01±0.95	31.27±1.38	30.72±0.65	38.58±0.86	32.17±0.45	29.25±0.71
20	37.53±1.42	32.81±2.96	29.66±1.56	39.04±0.71	30.47±0.50	30.50±1.02
60	63.41±0.78	20.27±1.26	16.32±1.23	63.68±0.63	19.29±0.23	17.03±0.86

Dox: doxycycline (1 μg/ml); CSSP: extract of cigarette sidestream smoke particulates

Fig. 7. Prolonged exposure of CL1-5(TO-AhR) cells to high concentrations of CSSP induced G0/G1 cell cycle arrest via the p53–p21–Rb1 signaling pathway. (A) The viability of CL1-5(TO-AhR) cells under 60 μg/ml CSSP treatment in the presence and absence of Dox-induced AhR expression was determined relatively to vehicle control ($n=4$). 1 μg/ml Dox was added 16 h before treatment started. Although AhR-induced cells exhibited higher viability in the beginning of the 72-h course, the viability of AhR-induced cells declined to a level similar to uninduced cells at the end. $^{*}p<0.05$ and $^{**}p<0.005$. (B) Cell cycle distribution after a 72-h treatment with 0–60 μg/ml CSSP was examined ($n=3$). A higher percentage of cells was located to the G0/G1 phase under 60 μg/ml CSSP, but there was no difference between AhR-induced and uninduced cells. (C) Expression of cell cycle regulatory genes in AhR-induced cells after a 72-h treatment with 60 μg/ml CSSP was profiled using a quantitative RT-PCR array and compared with vehicle control ($n=4$). The results of six regulatory genes were listed. (D) Changes of the p53–p21–Rb1 pathway were validated at protein level by Western blot analysis using antibodies against phosphor-p53 (P-p53), p21, total Rb1, and phosphor-Rb1 (P-Rb1). Protein expression of the house-keeping gene β-actin remained steady.

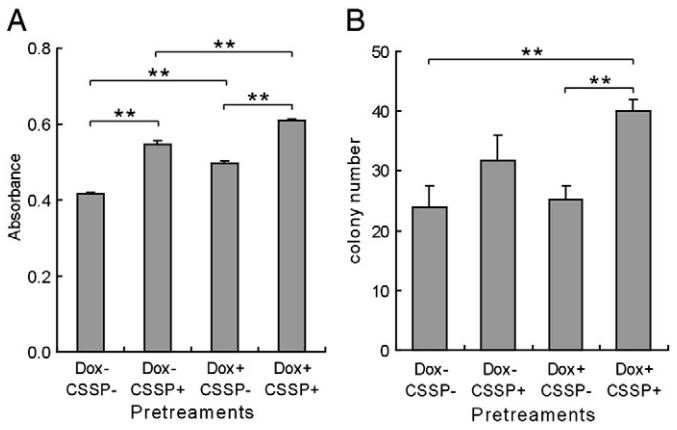


Fig. 8. CL1-5(TO-AhR) cells exhibited increased anchorage-dependent and independent proliferation when recovery from CSSP pretreatment. Cells were pretreated with vehicle or 60 μg/ml CSSP for 72 h before being seeded into CSSP-free liquid medium (A) or soft agarose medium (B). 1 μg/ml Dox was included in medium during and after exposure to induce AhR expression. Anchorage-dependent cell proliferation (A) and anchorage-independent colony formation (B) were determined 72 h and 5 weeks after seeding, respectively. * $p<0.05$ and ** $p<0.005$.

Although the mouse Nrf2 gene is a target gene for AhR (Miao et al., 2005), the human Nrf2 gene is unlikely to be directly regulated by AhR because no visible changes in Nrf2 protein level in CL1-5(TO-AhR) cells after Dox/CSSP treatment. AhR-induced and uninduced CL1-5(TO-AhR) cells also exhibited comparable Nrf2 activity and Nrf2 target gene expression (NQO1) under various CSSP conditions. Even with high CYP1B1 expression, AhR-overexpressing CL1-5(TO-AhR) cells did not generate substantial amounts of ROS to activate Nrf2 signaling after short periods of CSSP exposure. On the other hand, we found that UGT1A8 and UGT1A10 were concomitantly upregulated alongside CYP1B1 in AhR-overexpressing CL1-5(TO-AhR) cells. The concomitant upregulation suggests that AhR protects lung adenocarcinoma cells against oxidative stress by coordinate regulation of a set of phase I and II metabolizing genes that contain at least one AhR binding motif in the promoter region.

Although less ROS and DNA lesions were detected in AhR-overexpressing cells after short periods of CSSP exposure, AhR-signaled protection was not sufficient to shield cells from constant oxidative stress. Extension of the 60 μg/ml CSSP treatment to 72 h resulted in cell cycle arrest in the G0/G1 phase regardless of AhR expression level. It was likely that prolonged exposure led to cellular accumulation of macromolecular lesions. When damage went beyond a threshold, the p53–p21–Rb1 signaling pathway was activated. Consequently, genes responsible for S phase entry were repressed and cells were trapped in the G0/G1 phase (Lavin and Gueven, 2006; Sun et al., 2007). Cell cycle arrest seemed to prevent CSSP-exposed lung adenocarcinoma cells from undergoing apoptosis or senescence. Cells recommenced proliferation as soon as exposure was terminated.

The growth rate of CSSP-pretreated CL1-5(TO-AhR) cells was significantly increased as compared to vehicle pretreatment. AhR overexpression enhanced the stimulatory effect of CSSP pretreatment on cell growth. Meanwhile, AhR-overexpressing cells exhibited an increased capability to form colonies when recovery from CSSP pretreatment. Despite no effect on the DNA repair rate, AhR appeared to help cells overcome overall oxidative stress. During the defending process, AhR-overexpressing lung adenocarcinoma cells acquired higher clonogenicity. In this fashion, intermittent exposure such as the situation experienced by nonsmoking patients with a smoking spouse will greatly increase hyperplasia or the formation of tumors.

Overexpression of AhR in mouse embryonic fibroblast cells and human lung adenocarcinoma A549 cells also accelerated anchorage-dependent proliferation in the absence of exogenous ligands as what we observed with CL1-5(TO-AhR) (X. Chang et al., 2007; Shimba et al., 2002). AhR-overexpressing mouse fibroblasts had higher levels of cyclins and related kinases than AhR-deficient fibroblasts (X. Chang et al., 2007). In contrast, expression of cyclins and related kinases remained steady in human lung adenocarcinoma A549 cells regardless of AhR level. In A549 cells, AhR overexpression yielded an increase in E2F transcriptional activity, which was accompanied by upregulation of DNA replication regulators DP2, PCNA, and RFC38 (Shimba et al., 2002). On the other hand, AhR has been reported to synergize with Rb1 to inhibit E2F-dependent transcription and cell cycle progression by association with the E2F/Rb1 complex (Puga et al., 2000). Whether AhR actually stimulates adenocarcinoma cell proliferation through E2F needs to be verified.

In conclusion, we demonstrated that AhR could reduce CSSP-induced ROS production and oxidative DNA damage in lung adenocarcinoma cells. Although lengthened exposure to high concentrations of CSSP still caused sufficient stress to induce AhR-independent cell cycle arrest via the p53–p21–Rb1 signaling pathway, AhR-overexpressing lung adenocarcinoma cells exhibited higher proliferation and colony formation rates when recovery from exposure. Our results suggest that AhR plays a role in antioxidant defense and cigarette smoke-promoted clonogenicity in lung adenocarcinoma cells under the conditions of this study.

Conflict of interest statement

All authors declare no conflict of interest.

Acknowledgments

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References

Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C., Thomas, M., 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J. Immunol. 130, 1910–1917.

Chang, J.T., Chang, H., Chen, P.H., Lin, S.L., Lin, P., 2007a. Requirement of aryl hydrocarbon receptor overexpression for CYP1B1 up-regulation and cell growth in human lung adenocarcinomas. Clin. Cancer Res. 13, 38–45.

Chang, X., Fan, Y., Karyala, S., Schwemberger, S., Tomlinson, C.R., Sartor, M.A., Puga, A., 2007b. Ligand-independent regulation of transforming growth factor beta1 expression and cell cycle progression by the aryl hydrocarbon receptor. Mol. Cell. Biol. 27, 6127–6139.

Chen, D., Tian, T., Wang, H., Liu, H., Hu, Z., Wang, Y., Liu, Y., Ma, H., Fan, W., Miao, R., Sun, W., Wang, Y., Qian, J., Jin, L., Wei, Q., Shen, H., Huang, W., Lu, D., 2009. Association of human aryl hydrocarbon receptor gene polymorphisms with risk of lung cancer among cigarette smokers in a Chinese population. Pharmacogenet. Genomics 19, 25–34.

Cheng, Y., Chang, L.W., Cheng, L.C., Tsai, M.H., Lin, P., 2007. 4-Methoxyestradiol-induced oxidative injuries in human lung epithelial cells. Toxicol. Appl. Pharmacol. 220, 271–277.

Favreau, L.V., Pickett, C.B., 1991. Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. J. Biol. Chem. 266, 4556–4561.

Grimmer, G., Naujack, K.W., Dettbarn, G., 1987. Gaschromatographic determination of polycyclic aromatic hydrocarbons, aza-arenes, aromatic amines in the particle and vapor phase of mainstream and sidestream smoke of cigarettes. Toxicol. Lett. 35, 117–124.

IARC, 2004. Tobacco smoke and involuntary smoking. IARC monographys on the evaluation of the carcinogenic risk of chemicals to human. IARC, WHO, Lyon, France.

Kalthoff, S., Ehmer, U., Freiberg, N., Manns, M.P., Strassburg, C.P., 2010. Interaction between oxidative stress sensor Nrf2 and xenobiotic-activated aryl hydrocarbon receptor in the regulation of the human phase II detoxifying UDP-glucuronosyltransferase 1A10. J. Biol. Chem. 285, 5993–6002.

Kim, J.H., Kim, H., Lee, K.Y., Kang, J.W., Lee, K.H., Park, S.Y., Yoon, H.I., Jheon, S.H., Sung, S.W., Hong, Y.C., 2007. Aryl hydrocarbon receptor gene polymorphisms affect lung cancer risk. Lung Cancer 56, 9–15.

Kitamura, M., Kasai, A., 2007. Cigarette smoke as a trigger for the dioxin receptor-mediated signaling pathway. Cancer Lett. 252, 184–194.

Kohle, C., Bock, K.W., 2007. Coordinate regulation of Phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2. Biochem. Pharmacol. 73, 1853–1862.

Lavin, M.F., Gueven, N., 2006. The complexity of p53 stabilization and activation. Cell Death Differ. 13, 941–950.

Lee, H.L., Hsieh, D.P., Li, L.A., 2011. Polycyclic aromatic hydrocarbons in cigarette sidestream smoke particulates from a Taiwanese brand and their carcinogenic relevance. Chemosphere 82, 477–482.

Lin, P., Chang, H., Tsai, W.T., Wu, M.H., Liao, Y.S., Chen, J.T., Su, J.M., 2003. Overexpression of aryl hydrocarbon receptor in human lung carcinomas. Toxicol. Pathol. 31, 22–30.

Lin, T.C., Chien, S.C., Hsu, P.C., Li, L.A., 2006. Mechanistic study of polychlorinated biphenyl 126-induced CYP11B1 and CYP11B2 up-regulation. Endocrinology 147, 1536–1544.

Lin, S., Lin, C.J., Hsieh, D.P., Li, L.A., 2011. ERalpha phenotype, estrogen level, and benzo [a]pyrene exposure modulate tumor growth and metabolism of lung adenocarcinoma cells. Lung Cancer. (http://dx.doi.org/10.1016/j.lungcan.2011.08.010).

Lodovici, M., Akpan, V., Evangelisti, C., Dolara, P., 2004. Sidestream tobacco smoke as the main predictor of exposure to polycyclic aromatic hydrocarbons. J. Appl. Toxicol. 24, 277–281.

Ma, Q., Kinneer, K., Bi, Y., Chan, J.Y., Kan, Y.W., 2004. Induction of murine NAD(P)H:quinone oxidoreductase by 2,3,7,8-tetrachlorodibenzo-p-dioxin requires the CNC (cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon receptor) and Nrf2 signal transduction. Biochem. J. 377, 205–213.

Miao, W., Hu, L., Scrivens, P.J., Batist, G., 2005. Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. J. Biol. Chem. 280, 20340–20348.

Morabia, A., Wynder, E.L., 1991. Cigarette smoking and lung cancer cell types. Cancer 68, 2074–2078.

Olive, P.L., Banath, J.P., Durand, R.E., 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the “comet” assay. Radiat. Res. 122, 86–94.

Oyama, T., Sugio, K., Uramoto, H., Kawamoto, T., Kagawa, N., Nadaf, S., Carbone, D., Yasumoto, K., 2007. Cytochrome P450 expression (CYP) in non-small cell lung cancer. Front. Biosci. 12, 2299–2308.

Perng, D.W., Perng, R.P., Kuo, B.I., Chiang, S.C., 1996. The variation of cell type distribution in lung cancer: a study of 10,910 cases at a medical center in Taiwan between 1970 and 1993. Jpn. J. Clin. Oncol. 26, 229–233.

Puga, A., Barnes, S.J., Dalton, T.P., Chang, C., Knudsen, E.S., Maier, M.A., 2000. Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. J. Biol. Chem. 275, 2943–2950.

Radjendirane, V., Jaiswal, A.K., 1999. Antioxidant response element-mediated 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induction of human NAD(P)H:quinone oxidoreductase 1 gene expression. Biochem. Pharmacol. 58, 1649–1655.

Shimada, T., 2006. Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. Drug Metab. Pharmacokinet. 21, 257–276.

Shimba, S., Komiyama, K., Moro, I., Tezuka, M., 2002. Overexpression of the aryl hydrocarbon receptor (AhR) accelerates the cell proliferation of A549 cells. J. Biochem. 132, 795–802.

Sun, A., Bagella, L., Tutton, S., Romano, G., Giordano, A., 2007. From G0 to S phase: a view of the roles played by the retinoblastoma (Rb) family members in the Rb-E2F pathway. J. Cell. Biochem. 102, 1400–1404.

Tsou, T.C., Tsai, F.Y., Hsieh, Y.W., Li, L.A., Yeh, S.C., Chang, L.W., 2005. Arsenite induces endothelial cytotoxicity by down-regulation of vascular endothelial nitric oxide synthase. Toxicol. Appl. Pharmacol. 208, 277–284.

Wang, T.S., Chung, C.H., Wang, A.S., Bau, D.T., Samikkannu, T., Jan, K.Y., Cheng, Y.M., Lee, T.C., 2002. Endonuclease III, formamidopyrimidine-DNA glycosylase, and proteinase K additively enhance arsenic-induced DNA strand breaks in human cells. Chem. Res. Toxicol. 15, 1254–1258.

Yang, P.C., Luh, K.T., Wu, R., Wu, C.W., 1992. Characterization of the mucin differentiation in human lung adenocarcinoma cell lines. Am. J. Respir. Cell Mol. Biol. 7, 161–171.

Zangar, R.C., Davydov, D.R., Verma, S., 2004. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. Toxicol. Appl. Pharmacol. 199, 316–331.