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Involvement of Oxidative Stress and Activation of Aryl Hydrocarbon Receptor in Elevation of CYP1A1 Expression and Activity in Lung Cells and Tissues by Arsenic: An *In Vitro* and *In Vivo* Study

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Epidemiological evidence indicated that there was a synergistic interaction between arsenic and cigarette smoke on enhancement of lung cancer risk. Benzo[*a*]pyrene (B[*a*]P), a component in cigarette smoke, is one of the most carcinogenic compounds known. Animal studies have demonstrated that there were increased benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) adduct formation and lung tumorigenesis in animals when they were coexposed to B[*a*]P and arsenic. Since BPDE adduct is a by-product of B[*a*]P metabolism, elevation of B[*a*]P metabolism by arsenic is suspected. However, the effects of arsenic on cytochrome P450 1A1 (CYP1A1) status (expression and activity), which is essential for B[*a*]P metabolism, either in lung cells or in lung tissues, are never demonstrated. We hypothesized that arsenic would enhance aryl hydrocarbon receptor (AhR) activation leading to CYP1A1 expression and activity in lung cells. Indeed, our present study successfully demonstrated the elevation of *CYP1A1* messenger RNA expression in H1355 cells, a human lung adenocarcinoma cell line, as well as CYP1A1 expression and activity in lung tissues of arsenic-exposed mice. We further demonstrated that this elevation of *CYP1A1* expression could be effectively blocked with AhR antagonist, 3′,4′-dimethoxyflavone, indicating that the arsenic-induced CYP1A1 expression and activity were via AhR activation. Furthermore, we found that arsenic-induced AhR activation and -enhanced CYP1A1 expression can be further increased by a prooxidant, buthionine-(*S*,*R*)-sulfoximine, and suppressed by antioxidants, such as N-acetylcysteine and catalase. Our findings provided clear evidence that arsenic can enhance CYP1A1 expression and activity via AhR activation, and the arsenic-induced AhR activation is probably triggered by oxidative stress.

Key Words: **arsenic; cytochrome P450 1A1; oxidative stress; aryl hydrocarbon receptor.**

Epidemiological studies demonstrated that cigarette smokers in arseniasis areas have significant higher lung cancer risk than those in the general population (Chen *et al*., 2004; Ferreccio *et al*., 2000). A recent review (Mead, 2005) also stated that evidence for synergy between cigarette smoke and arsenic in lung carcinogenesis is mounting. The biological mechanisms for this synergistic effect are probably complex and multifaceted. We have recently reported that altered p53 status by cigarette smoke could serve as a platform for arsenic to promote cigarette smoke–induced carcinogenesis (Liao *et al*., 2007). We have also reported that arsenic can further promote the carcinogenic potential of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a component in cigarette smoke, by enhancement of the metabolism of NNK (Lee *et al*., 2007). Our present investigation is focused on the exploration on the interaction between arsenic and benzo[*a*]pyrene (B[*a*]P), another important carcinogenic component in cigarette smoke.

Cigarette smoke is a complex mixture of many chemicals (Green and Rodgman, 1996). Among which, more than 60 compounds are known to be carcinogenic (Hecht, 2003). Polycyclic aromatic hydrocarbons (PAHs) constitute the major group of organic carcinogens in cigarette smoke (Hecht, 2003). The carcinogenicity of many PAHs is closely associated with their metabolic activation. The metabolic activation of these PAHs, including that of B[*a*]P, is catalyzed by biotransformation of these chemical compounds via cytochrome P450 (CYP) enzymes (Shimada and Fujii-Kuriyama, 2004). Cytochrome P450 1A1 (CYP1A1) is a major CYP enzyme in many other extrahepatic tissues, including lung, for metabolic activation. Many metabolic products or by-products from these PAH compounds, unfortunately, are carcinogenic (Buters *et al*., 1999; Shimada and Fujii-Kuriyama, 2004; Shimizu *et al*., 2000; Spink *et al*., 2002; Spivack *et al*., 1997). Since the carcinogenicity of B[*a*]P is heavily depended on its metabolic product benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE), it is reasonable to believe that arsenic may also increase B[*a*]P carcinogenicity via enhancement of B[*a*]P metabolism. Indeed, it has been demonstrated that cotreatment of arsenic and B[*a*]P

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significantly increased formation of B[*a*]P epoxide-DNA adducts (BPDE adducts) in lung tissues of mice (Evans *et al*., 2004). Furthermore, increased lung tumorgenesis in mice and in hamsters was also observed when B[*a*]P-treated animals were cotreated with arsenic (Ishinishi *et al*., 1977; Pershagen *et al*., 1984).

CYP1 enzyme, especially CYP1A1, is known to be a key metabolic enzyme in B[*a*]P metabolism (Shimada and Fujii-Kuriyama, 2004). Therefore, it becomes reasonable to postulate that an increased B[*a*]P metabolism by arsenic is resulted from enhancement of CYP1A1 status (expression and activity). Indeed, it has been demonstrated *in vitro* (hepatocyte cultures) that arsenic could induce an elevation of CYP1A1 messenger RNA (mRNA) levels within 24 h (Chao *et al*., 2006; Elbekai and El-Kadi, 2005; Kann *et al*., 2005). Since the lung appears to be the prime target organ for arsenic/cigarette smoke interaction and influence of arsenic may be cell or tissue specific, study using lung cells (*in vitro*) and lung tissues (*in vivo*) under more prolonged exposure conditions is needed. Our present study is therefore designed with these objectives in mind. There are ample reports in the literature denoting increased B[*a*]P metabolites and BPDE adducts in animals coexposed to B[*a*]P and arsenic (Evans *et al*., 2004); thus, there is no need for us to duplicate these observations in our present study. We will focus our research efforts to provide the needed mechanistic explanation for the enhancement of B[*a*]P metabolism by arsenic as reported by others in previous studies.

MATERIALS AND METHODS

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from ULTRA Scientific (Kingeston, RI) and dissolved in dimethyl sulfoxide before use. Sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Formalin and xylene were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Other chemicals, including sodium arsenite ($NaAsO_2$), 3′,4′-dimethoxyflavone (DMF), catalase, N-acetylcysteine (NAC), and buthionine-(*S*,*R*)-sulfoximine (BSO), used in the present study were obtained from Sigma Co. (St Louis, MO). Sodium arsenite was dissolved in double-distilled water (d_2H_2O) before use.

Animal treatment. Young (6–7 weeks old) male ICR mice, with average body weight of 30 g, were used in the present study. Animals were housed in the animal facilities of the National Health Research Institutes (NHRI) in accordance with standard and approved animal husbandry practice and regulation. All animal treatments and experimental protocol for this study have been reviewed and approved by the Animal Control Committee at the NHRI to ensure that all animals were treated humanely without undue sufferings.

Animals were randomly divided into three groups of six ($n = 6$ per group). Two groups of the animals were daily gavaged with arsenic ($NaAsO_2$) at doses of 10 or 20 mg/kg body weight for 10 days. The third group of animals served as controls and was gavaged similarly with equal volumes of double-distilled water. The health conditions (body weight, growth rates, fur conditions, general behavior, etc.) were monitored daily. At sacrifice, lungs of the animals were sampled, weighed, and stored frozen at − 80°C.

Arsenic analysis. Lung tissues were then digested with 5 ml of nitric acid and 1 ml of hydrogen peroxide followed by microwave digestion. Arsenic content in the tissues was determined with inductively coupled plasma-mass spectrometry (MS) (Elan6100; PerkinElmer, Shelton, CT).

Cell culture. H1355 cells, a human lung adenocarcinoma cell line (American Type Culture Collection, Manassas, VA), were used in our study. These cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum in a 37°C incubator with a humidified mixture of 5% CO_2 and 95% air. The medium was changed twice a week, and cells were passaged by trypsinization every week.

Cell viability assay. H1355 cells were seeded in a 96-well plate at a density of 2×10^5/well and cultured in supplemented RPMI 1640 medium. After incubating for 48 h, cells were treated with 1% d_2H_2O or 1, 5, or 10μM $NaAsO_2$ for further 72 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (Tsou *et al*., 2004).

Treatment of cultivated cells. H1355 cells were seeded in six-well plates in the same density for 48 h. When treatment began, cells were incubated with double-distilled water (d_2H_2O) or 10μM $NaAsO_2$ for 24, 48, or 72 h for time course study and 1% d_2H_2O and 1 or 10μM $NaAsO_2$ for 72 h for dose-response study. Additionally, to determine the effects of other chemicals (DMF, NAC, BSO, and catalase), cells were incubated with 1% d_2H_2O, chemical, 10μM $NaAsO_2$, and 10μM $NaAsO_2$/chemical concomitantly for 72 h. Concentration of each chemical used in this study was as follows: DMF 10μM, NAC 2mM, BSO 1μM, and catalase 1000 units/ml. For our "oxidative stress study," the cells were pretreated with NAC or BSO for 30 min or catalase for 2 h before exposing to arsenic. At the end of all treatments, cells were harvested immediately for RNA preparation. In the present study, 1nM TCDD was used as the positive control for the induction of *CYP1A1* expression.

Transient transfection and luciferase reporter assay. The reporter vector, 4× dioxin-response element (DRE)-TATA-Luc, was kindly provided by Dr Lih-Ann Li and Dr Tsui-Chun Tsou (NHRI, Taiwan, People's Republic of China). A plasmid expressing the bacterial β-galactosidase gene and serving as an internal control of transfection efficiency was kindly provided by Dr Gwo-Tarng Sheu (Chung Shan Medical University, Taiwan, People's Republic of China). H1355 cells were seeded at the density of 1×10^5/well and incubated for 24 h. Then, cells were transiently transfected with the 4× DRE-TATA-Luc reporter vector and the transfection efficiency control vector using Lipofectamine reagent (Invitrogen Corporation, Carlsbad, CA) and incubated for 6 h before treatments began. H1355-DRE-Luc cells were treated with 1% d_2H_2O or 10μM $NaAsO_2$ for 24, 48, or 72 h for time course study. Exposure of H1355-DRE-Luc cells to 10μM $NaAsO_2$ with or without 2mM NAC or 1μM BSO for 72 h was also conducted. Cells were harvested for luciferase activity measurement and β-galactosidase activity determination. Cell lysate for luciferase activity determination was prepared using Luciferase Assay System (Promega, Madison, WI), and luminescence was detected by programmed microplate luminometer MicroLumatPlus (LB96V; EG&G, Berthold, Germany) with excitation and emission wavelengths at 400 and 460 nm, respectively. For β-galactosidase activity determination, cell lysate was incubated with assay buffer (200mM $NaPO_4$, pH 7.0–7.3, 2mM $MgCl_2$, 100mM β-mercaptomethanol, and 4.4mM O-nitrophenyl B-D-galactopyranoside) of the same volume for 30 min at 37°C, followed by measuring the absorbance at 420 nm. DRE activity was defined as relative luminescence unit/absorbance at 420 nm.

Quantitative real-time reverse transcriptase-PCR assay. Total RNA of cells or lung tissues was prepared using the TRIZOL reagent (Life Technologies, Rockville, MD) and the phenol-chloroform extraction method. Synthesis of cDNA was performed by 2 μg total RNA mixed with 250 ng random primer (BioLabs, Beverly, MA) using moloney murine leukemia virus reverse transcriptase (Promega). Quantitative PCR was carried out using the "TagMan" gene expression kit (PerkinElmer Applied Biosystem, Foster City, CA) and analyzed on an ABI PRISM 7900 Sequence Detector System (PerkinElmer Applied Biosystem). Primers and probes were either designed with the assistance of the computer program—Primer Express or from the

Assay-on-Demand gene expression assay mix (PerkinElmer Applied Biosystem). These sequences of primers and probes are listed in Table 1. Quantitative values were obtained from the threshold cycle (Ct) number, the increase in signal being associated with an exponential growth for PCR product when detected. Each sample target gene expression level was normalized to its *GAPDH* mRNA content. The relative mRNA levels $= 2^{-\Delta Ct}$, $\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$.

Microsome preparation. Tissues were rinsed with ice-cold PBS before preparing microsomes, followed by homogenizing with 50mM KH_2PO_4/K_2HPO_4 (pH 7.4) on ice. Homogenates were centrifuged at 4°C, 13,000 × g for 20 min, and then the supernatants were further ultracentrifuged at 4°C, 104,000 × g for 1 h. Microsomal pellets were resuspended in Tris-sucrose buffer (50mM Tris, 250mM sucrose, pH 8.0) and stored at – 80°C until analysis.

Ethoxyresorufin-O-deethylase activity assay with liquid chromatography/MS. CYP1 enzyme activity was measured as ethoxyresorufin-O-deethylase (EROD) activity. 7-Ethoxyresorufin (2μM) was used as the substrate for CYP1A1 (Burke *et al*., 1994). The assay was performed in a final volume of 0.2 ml containing 1mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 3mM $MgCl_2$, and 79mM potassium phosphate buffer, pH 7.4. Incubation was carried out aerobically at 37°C. At the end of 30-min incubation, 80 μl of ice-cold acetonitrile containing the internal standard (chlorzoxazone; 100 ng/ml) was added into each incubation mixture to terminate the reaction. The samples were then vortexed and centrifuged at 20,800 × g for 20 min at room temperature. The obtained supernatant was used for the analysis of resorufin formation by liquid chromatography (LC)/MS. The LC/MS system consisted of an Agilent 1100 Series LC System and Series mass spectrometer (Palo Alto, CA). The column used to analyze the incubation mixtures was Zorbax Eclipse XDB-C_8 (5 μm, 150 × 3.0 mm inner diameter, Agilent Technologies, Santa Clara, CA). Mobile phase consisted of solvent A (10mM ammonia acetate containing 0.1% formic acid) and solvent B (acetonitrile). The flow rate was 0.5 ml/min. The gradient systems used to separate resorufin and chlorzoxazone were 30% B to 50% B (0–1 min), 50% B to 98% B (1–3 min), 98% B (3–9 min), 98% B to 30% B (9–10 min), and 30% B (10–15 min). The column temperature was set at 25°C. The retention times were resorufin/chlorzoxazone, 6.7/7.0 min. Data acquisition was via selected ion monitoring. Ions representing the $(M\text{-}H)^-$ species for the metabolite were selected, and the peak areas were measured. The concentration of resorufin formed during microsomal incubation was calculated by comparing the peak area ratio of the testing samples with that of a known concentration of metabolite in microsomal incubations in the absence of NADPH.

Immunohistostaining for CYP1A1. Lung tissues were fixed with 10% buffered formalin for 16–48 h and then subjected to a standard histopathological tissue processing. Lung tissue sections were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and then microwaved in 0.01M citrate buffer (pH 6.0) for 5 min for three times. After washing, the sections were incubated at 4°C overnight with anti-CYP1A1 (1:100 dilution, clone H-70; Santa Cruz Biotechnology, Santa Cruz, CA) and followed by a standard staining procedure using the LSAB kit (DakoCytomation, Glostrup, Denmark).

Light microscopic assessment of the immunostaining sections was performed by two independent observers (Dr H. Chang and Dr J.-P. Wu). Immunostaining results were scored independently and compared at the end of assessment. Image-analyzing software (Metamorph ver. 7.0; Molecular Devices Corporation, Downingtown, PA) was applied for bias-free determination of cases into negative or positive according to the staining intensity and number of stained cells. CYP1A1 showed a cytoplasmic staining. Positive expresser was defined as stained cells showing that CYP1A1-positive immunoreactivity and their cytoplasmic staining intensity were stronger than adjacent stroma. We found that pulmonary CYP1A1-positive cells presented in bronchial and bronchiolar epithelial cells, while types I and II alveolar cells were CYP1A1 negative. Because bronchus did not present in all studied lung sections, the percentage of CYP1A1-positive cells was calculated in the bronchiole. The percentage of CYP1A1-positive cells was calculated by dividing the number of CYP1A1-positive cells with the total number of examined bronchiolar cells in each tissue section.

Statistic analysis. Comparison of the results between various experimentally treated groups and their corresponding controls was carried out by Student *t*-test. All comparisons were considered significantly different when $p < 0.05$.

TABLE 1
Sequences of Primers and Probes

Gene	Sequences
Human	
CYP1A1	F: 5′-CAC CAT CCC CCA CAG CAC-3′
	R: 5′-ACA AAG ACA CAA CGC CCC TT-3′
	Probe: 5′-ACC AGC AAC CCT GCC AGC AA-3′
AHR	F: 5′-AGA GTC TGG ACA AGG AAT TGA A-3′
	R: 5′-GAA GTG GAG TAG CTA TCG CAA A-3′
	Probe: 5′-TTC CCT TGG AAA TTC ATT GCC AGA A-3′
GAPDH	F: 5′-GCA CCG TCA AGG CTG AGA AC-3′
	R: 5′-TGA TGA TCT TGA GGC TGT TGT CA-3′
	Probe: 5′-CCC ATC ACC ATC TTC CAG GAG CGA-3′
Mouse	
CYP1A1	F: 5′-CCT CTT TGG AGC TGG GTT T-3′
	R: 5′-CGA TCT CTG CCA ATC ACT GT-3′
	Probe: 5′-TCT CGT GGA GCC TCA TGT ACC TGG-3′
Gapdh	Assay-on-Demand

RESULTS

Effects of Arsenic on Pulmonary CYP1A1 Expression and Activity in Mice

ICR mice were fed with sodium arsenite in distilled water via daily gavage at doses of 0, 10, or 20 mg As/kg body weight for 10 days. Both pulmonary CYP1A1 mRNA levels and activities in animals fed with 10 and 20 mg/kg As showed dose-correlated increases of 4.0- and 4.7-fold of control ($p < 0.05$), respectively (Figs. 1A and 1B). Arsenic analysis further revealed that tissue accumulation of arsenic in lungs also increased in a dose-correlated manner. Approximately 3.2-fold more arsenic was found in lung tissues of animals treated with 20 mg/kg As than those from lower dosing groups (10 mg/kg As) (Fig. 1F). Thus, it appears that there was a correlation of high tissue accumulation of arsenic with higher CYP1A1 status (expression and activity).

We have also demonstrated the elevation of expression of CYP1A1 proteins in lung tissues with CYP1A1 immunohistochemistry. CYP1A1 immunostaining was found to be primarily located in nonciliated bronchiolar epithelial cells (Fig. 1C). This CYP1A1 protein (positive immunostaining) was significantly increased in arsenic-treated animals (Fig. 1D). Quantitative cell counting via morphometric analysis revealed that the percentage of CYP1A1-positive cells in the bronchiolar epithelium increased from 24.1% in controls to 34.2% in arsenic-treated mice (Fig. 1E) ($p < 0.05$). These results provided important histochemical demonstrations showing that CYP1A1 protein expression was upregulated in very specific loci (bronchiolar epithelial cells) in the lung by arsenic.

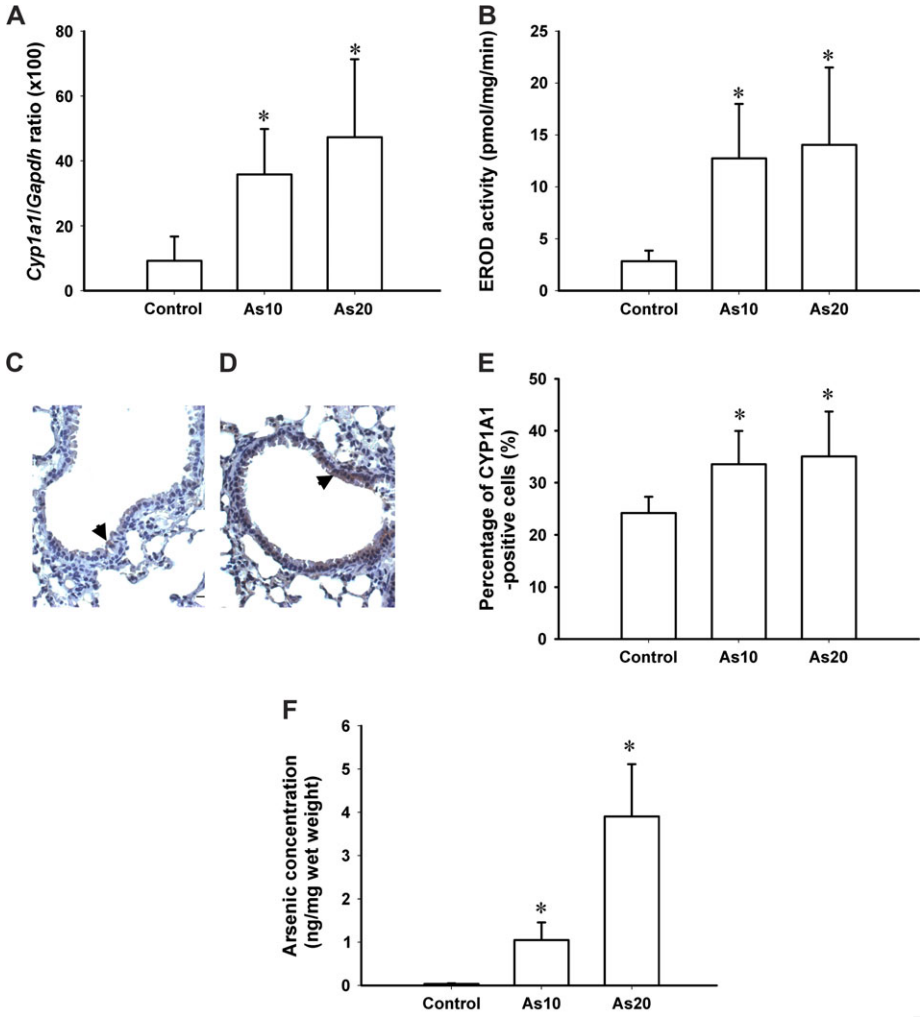

FIG. 1. Effects of arsenic on pulmonary *CYP1A1* expression, CYP1A1 (EROD) activity, CYP1A1 protein expression and distribution, and arsenic distribution in male ICR mice which were daily gavaged with 0, 10, or 20 mg As/kg for 10 days. (A) Significant elevations in *CYP1A1* mRNA (3.9- to 5.1-fold) by arsenic were demonstrated *in vivo*. (B) Increases in CYP1A1 enzyme activities *in vivo* in lung tissues (about 4.5- to 5.0-fold) were also observed. (C, D) Immunostaining for CYP1A1 protein labeled the CYP1A1-positive cells (bronchiolar epithelial cells) with brownish coloration in lungs of control (C) and arsenic-treated animals (D). An increase in immunostaining was noted in the bronchiolar epithelium of arsenic-treated animals denoting an increase in CYP1A1 protein in these cells after arsenic treatment. (E) Quantitative cell counts also revealed significant increases in CYP1A1-positive cells from 24.1% (controls) to 34.0% in the bronchiolar epithelium of arsenic-exposed mice. (F) Arsenic analysis showed that there was a definitive dose-corresponded increase in tissue arsenic in the lung tissues of arsenic-treated animals (3.2-fold more arsenic in the As 20 animals than in the As 10 animals). Results were presented as mean ± SD. Each mean was the average of six animals. *$p < 0.05$ as compared with controls. Arrow indicates the location of CYP1A1-positive cells.

Effects of Arsenic on Cell Growth, mRNA Levels of CYP1A1, and Aryl Hydrocarbon Receptor in H1355 Cells

Despite a slight reduction (10%) of cell growth when the cells (H1355) were treated with 10μM of arsenite (Fig. 2A), the overall survivability of the cells was still good showing that the cytotoxicity of arsenic for these cells are only minimal at these arsenic dose levels. Under these subtoxic conditions, the cellular CYP1A1 mRNA levels not only have not reduced but also were actually significantly elevated both in dose- and time-dependent manners when the cells were treated with 10μM $NaAsO_2$ ($p < 0.05$) (Figs. 2B and 2C). In our present study, we note that the arsenic-induced elevation of CYP1A1 mRNA levels was not accompanied by an increase in aryl hydrocarbon receptor (AhR) mRNA levels (Fig. 2D). Thus, it is apparent that the induction of increase in CYP1A1 mRNA levels by arsenic was accomplished independently from enhancement of *AhR* gene expression.

Effects of AhR Antagonist on the Induction of CYP1A1 mRNA Expression by Arsenic in H1355 Cells

While we have revealed that arsenic-induced elevation in *CYP1A1* expression was not accompanied by changes in *AhR* expression, we still need to clarify if AhR activity was affected or not under our experimental conditions. DMF is an AhR

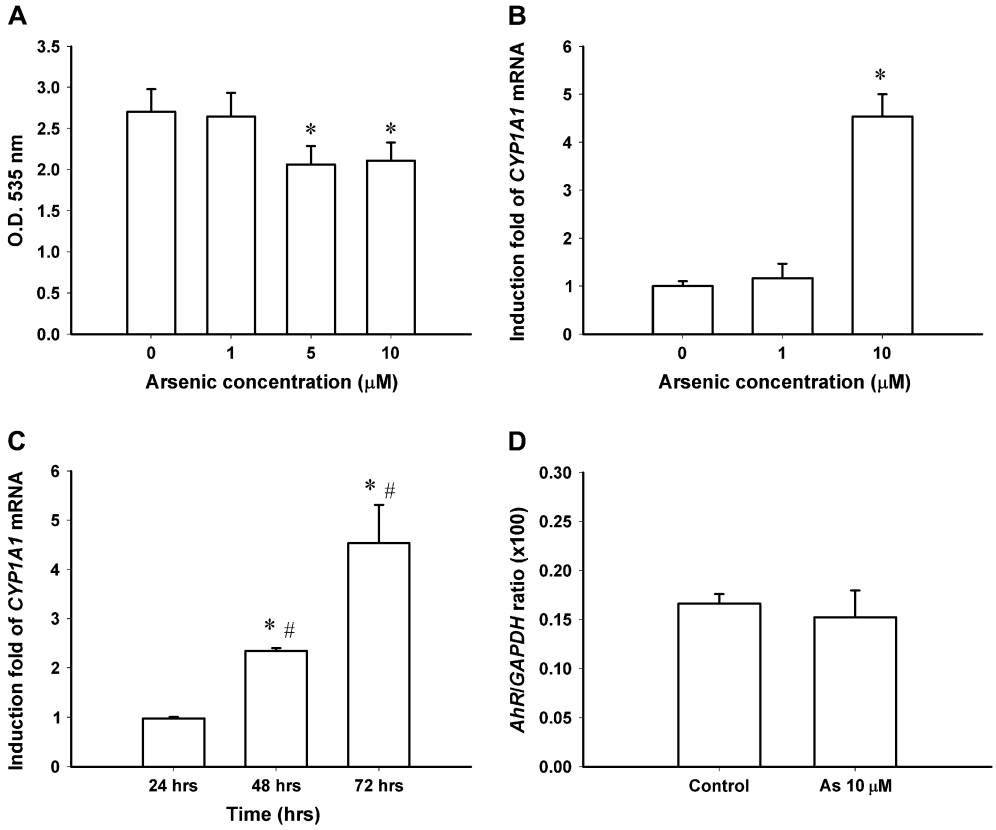

FIG. 2. Effects of arsenic on cell viability, the mRNA levels of CYP1A1, and AhR in H1355 cells. (A) The cell growth rate was only slightly affected (10%) by arsenic showing that the arsenic concentrations used in our present study only gad minimal cytotoxicity. (B, C) There was a dose- and time-corresponded increase in CYP1A1 mRNA induction in arsenic-treated H1355 cells *in vitro*. (D) No change in AhR expression was observed in arsenic-treated H1355 cells. These studies showed that arsenic enhanced CYP1A1 mRNA induction without change in AhR expression. Data are presented as mean ± SD. Mean was the average of four replicates. $^{*}p < 0.05$ compared with d_2H_2O-treated cells. $^{\#}p < 0.05$ compared to the results of 24-h treatment.

antagonist that is a known inhibitor for AhR activity (activation) (Lee and Safe, 2000). Our study showed that inhibition of AhR activity by DMF effectively blocked inductions of CYP1A1 mRNA levels by arsenic and by TCDD (used as the positive control) ($p < 0.05$) (Fig. 3A). These results imply that arsenic-induced elevation in CYP1A1 mRNA levels is still via AhR activation.

Effects of Arsenic on DRE Activity in H1355 Cells

TCDD, like many other classic AhR ligands, significantly increased DRE activity in H1355-DRE-Luc cells after exposure (Fig. 3B). This induction is usually rapid (within 24 h). A time-delayed (48–72 h) elevation of DRE activity, however, was observed in cells treated with 10μM $NaAsO_2$. This time-delayed activation of AhR by arsenic suggests that arsenic does not behave like a classic AhR agonist and may trigger AhR activation directly or indirectly in an unconventional manner.

Effects of Oxidative Status Modulators on Induction of CYP1A1 mRNA Levels and DRE Activity by Arsenic in H1355 Cells

In order to determine whether arsenic-induced oxidative stress was involved in the enhancement of *CYP1A1* expression, we need to demonstrate not only suppressions of arsenic-induced enhancements with antioxidants (NAC and catalase) but also further promotions of such enhancements by a prooxidant (BSO). We found that treatments with either NAC or catalase effectively inhibited the arsenic-induced elevation in *CYP1A1* mRNA levels (Figs. 4A and 4C). Furthermore, treatment with BSO significantly enhanced the arsenic-induced *CYP1A1* expression even further (Fig. 4E). Similar phenomena were demonstrated with DRE activation when H1355-DRE-Luc cells were treated with arsenic and different oxidative modulators: NAC, catalase, or BSO (Figs. 4B, 4D, and 4F). These findings provide compelling evidence that oxidative stress is involved in the arsenic-induced elevation in AhR activation (DRE activity) leading to an increased CYP1A1 status (expression and activity).

DISCUSSION

Several epidemiological studies have provided strong indications that there is a malignant synergy between arsenic exposure and cigarette smoking leading to enhanced lung carcinogenic risk in humans (Chen *et al*., 2004; Ferreccio *et al*., 2000).

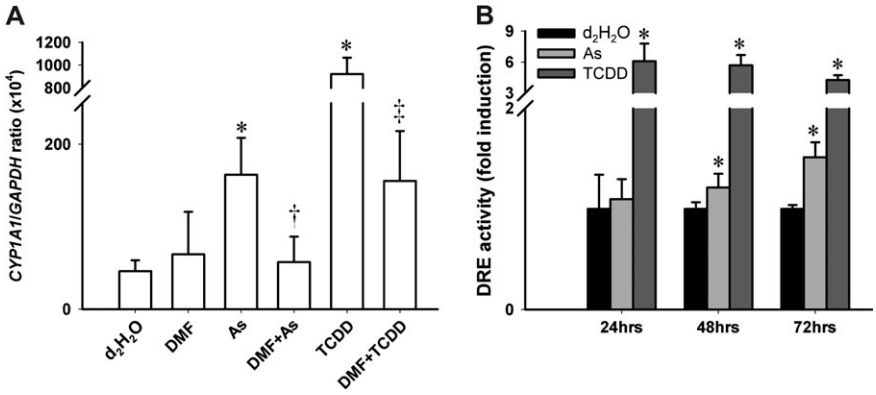

FIG. 3. Effects of AhR antagonist, DMF, on CYP1A1 expression and DRE activity in H1355 cells. (A) The induction of CYP1A1 expression in H1355 cells by arsenic was totally nullified by DMF, a known blocker for AhR activity (activation), indicating that arsenic-induced enhancement of CYP1A1 expression was via AhR activation. TCDD was used as a positive control for CYP1A1 mRNA expression in this study. (B) Significant time-dependent elevation of DRE activity (AhR activity) in H1355-DRE-Luc cell cultures, although much less than that induced by TCDD (positive control), could be demonstrated in lung cells after arsenic treatment. These studies, together with those illustrated in Figure 2, showed that arsenic could enhance CYP1A1 expression via AhR activation without elevation of AhR expression. Data are presented as mean ± SD. Mean was the average of four replicates. $^{*}p < 0.05$ compared with d_2H_2O-treated cells. $^{\dagger}p < 0.05$ compared with As alone–treated cells. $^{\ddagger}p < 0.05$ compared with TCDD-treated cells.

A recent review by Mead (2005) further stated that "the lung seems to be a major site of action of ingested arsenic . . . arsenic does not always operate alone. Rather, arsenic appears to work with other factors to promote factor . . . The classic cofactor in this regard may be tobacco smoke. There are mounting evidence of a malignant synergy between smoking and arsenic . . . smokers are at an increased risk from arsenic in drinking water and appears to comprise a susceptible subpopulation." Indeed, animal studies have also demonstrated that arsenic enhanced the carcinogenic potential of B[*a*]P, a potent carcinogenic compound found in cigarette smoke (Hecht, 2003) via increases in B[*a*]P metabolites and BPDE adduct formation (Evans *et al*., 2004; Ishinishi *et al*., 1977; Pershagen *et al*., 1984). Thus, it becomes reasonable to assume that enhancement of metabolic activation of B[*a*]P by arsenic is critical in this process. However, the precise mechanism underlying such metabolic enhancement of B[*a*]P by arsenic is still unknown.

B[*a*]P is a potent AhR agonist. Its metabolism is via increased CYP1A1 enzyme expression and activity for metabolic breakdown of B[*a*]P (Shimada and Fujii-Kuriyama, 2004). Induction of *CYP1A1* expression by arsenic has been reported in arsenic-treated hepatoma cell cultures within 24 h (Chao *et al*., 2006; Elbekai and El-Kadi, 2005; Kann *et al*., 2005). Results from our present study with human lung epithelial cells were consistent with those findings in hepatocytes. We further demonstrated that the elevation of *CYP1A1* expression in human lung epithelial cells was long lasting. Our *in vivo* study also affirmed that arsenic exposure induced significant elevations in CYP1A1 status (expressions and activity) in lung tissues in a dose-correlated manner. Specific immunohistochemical method for CYP1A1 protein further demonstrated increases both in the staining intensity and in the number of stain-positive cells (bronchiolar epithelial cells) in lungs after arsenic exposure. In addition to *in vitro* confirmation, our present study also provided the first and valuable demonstration that arsenic induced elevation in CYP1A1 status in lung tissues of animals exposed to arsenic. It is apparent that arsenic can induce CYP1A1 enhancement by "triggering" AhR activation without altering *AhR* expression. This triggering of AhR activation by arsenic may be made indirectly either via factors such as reactive oxygen species (ROS) production, oxidative stress, or other metabolic by-products generated by arsenic or via direct interaction between arsenic and AhR.

Arsenic is a known metalloid effectively inducing oxidative stress with disturbance in the intracellular glutathione (GSH) status and generation of ROS (Pi *et al*., 2003; Rin *et al*., 1995; Tsou *et al*., 2004; Valko *et al*., 2005). More importantly, *in vivo* study with Syrian golden hamsters demonstrated that arsenic and cigarette smoke synergistically increased oxidative stress in lungs (Hays *et al*., 2006). Furthermore, using Hepa 1c1c7 cells, Elbekai and El-Kadi (2005) demonstrated a concomitant elevation of *CYP1A1* expression and oxidative status when these cells were treated with arsenic. Since increased oxidative stress and elevated CYP1A1 status may simply occur simultaneously without causal relationship to each other, such causal relationship, if exists, needs to be demonstrated. In our study, we have successfully demonstrated this important causal relationship by showing that the enhancement of the arsenic-induced *CYP1A1* status can be further promoted with a prooxidant (BSO) and significantly suppressed with antioxidants (NAC and catalase). Furthermore, recent studies revealed that AhR-aryl hydrocarbon receptor nuclear translocator (ARNT) is highly sensitive to arsenic, and there is some indication that arsenic may influence AhR-ARNT and the modification of AhR-dependent gene expression is associated with oxidative stress induced by arsenic (Kitchin and Wallace, 2008; Nelson *et al*., 2007).

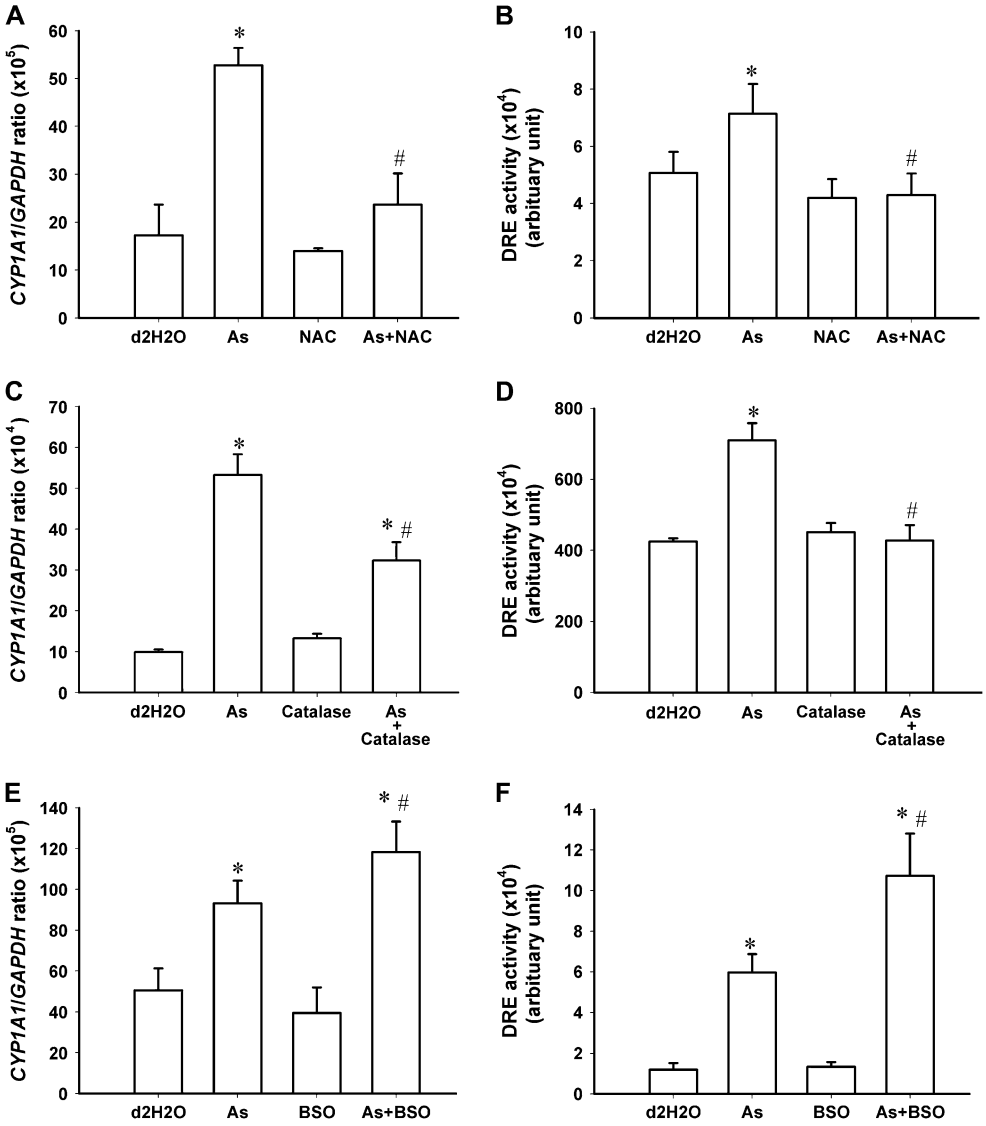

FIG. 4. Effects of oxidative status modulators, NAC, catalase, and BSO, on the induction of CYP1A1 expression and DRE activity in H1355 cells. Arsenic-induced CYP1A1 expression and DRE activities could be effectively suppressed by antioxidants NAC (A, B) and by catalase (C, D) and could be further promoted by the prooxidant BSO (E, F). These findings affirmed the involvement of oxidative stress in arsenic-induced activation of AhR and enhancement of CYP1A1 expression. Data are presented as SEM. Mean was the average of four replicates. *$p < 0.05$ compared with cells treated with arsenic alone.

The observations that we have made in our studies with NAC and with BSO were intriguing. NAC is known to replenish cellular GSH and, thus, may promote antioxidation. BSO, on the other hand, is an inhibitor of the glutamatecysteinyl ligase enzyme and, thus, may exhibit a "prooxidative" action. In our study, if arsenic acts by elevating oxidative stress via a GSH-dependent mechanism, inhibition of GSH synthesis by BSO alone should cause similar effect. However, this was not apparent in our findings (Fig. 4). Thus, possibility exists that arsenic could be bound and sequestered by NAC for reducing the arsenic effect and not as much bound by the lowered GSH levels in BSO-treated cells. Furthermore, although adding catalase into the medium also reduced arsenic-induced effects, this reduction phenomenon may be induced extracellularly rather than intracellularly because the molecular size of catalase may be too large to enter the cells freely for intracellular antioxidative actions. It is possible that interactions or reactions between heme iron and phenolic or alcohol groups in the medium may contribute to the effects that we have observed. Future studies will be needed to further clarify and elucidate these mechanisms.

Arsenic is known to induce the production of heme oxygenase-1 (HO-1) via oxidative stress (Elbekai and El-Kadi, 2005). HO-1 will in turn catalyze the breakdown of heme into bilirubin, which is shown to be an endogenous AhR ligand (Albores *et al*., 1989; Elbekai and El-Kadi, 2005; Sinal and Bend, 1997). Thus,

possibility certainly exists that the triggering of AhR activation by arsenic is attributed to or contributed by increased bilirubin production by arsenic. Further research obviously is still needed to affirm this possibility.

Aside from oxidative stress–based mechanisms, the possibility of direct interaction between AhR and arsenic or its metabolites should also be considered. There are indications that the sulfhydryl (thiol) groups in AhR play an important role in the binding of ligands to AhR as well as in the binding of AhR to DNA (Denison *et al*., 1987; Ireland *et al*., 1995; Xu *et al*., 1998). Arsenic has been reported to react with thiol groups in proteins resulting in structural changes and functional alterations of these thiol-rich proteins or enzymes (Aposhian, 1989; Valko *et al*., 2005). Thus, it is also plausible that arsenic or its metabolites may bind directly to AhR triggering its activation via alterations of the sulfhydryl moiety of AhR. The mechanistic base for arsenic enhancement of B[*a*]P metabolism is obviously complex. In the present study, we can only offer our postulated thoughts on interaction between arsenic and AhR for such enhancement. These thoughts, we hope, will inspire future investigations.

Although high levels of arsenic are detected in the urine of humans in arseniasis areas, arsenic levels in the lung of humans in arseniasis areas are not available. There are two arseniasis areas in Taiwan: blackfoot disease (BDF)–endemic areas and Lanyang Plain. Recently, a physiologically based pharmacokinetic-based study predicted that arsenic concentrations in the lung ranged from 3.76 to 9.46 ng/mg in humans of BDF-endemic areas and 8.23 to 19.92 ng/mg in humans of Lanyang Plain in Taiwan (Ling and Liao, 2007). In our present study, arsenic concentrations in the lungs of arsenic-treated mice were 1–4 ng/mg. Therefore, elevation of CYP1A1 expression and activity may also occur in the lungs of humans in arseniasis areas.

While both epidemiological and laboratory studies provided strong indications that there is a synergistic interaction between cigarette smoke or B[*a*]P and arsenic in enhancement of B[*a*]P metabolism and increased lung cancer risk (Chen *et al*., 2004, Chiu *et al*., 2004; Evans *et al*., 2004; Ishinishi *et al*., 1977; Pershagen *et al*., 1984), our present study has provided important information on the interrelationship between arsenic, oxidative stress, AhR activation, and enhancement of CYP1A1 expression/activity which certainly helps provide the mechanistic base for the reported increases in B[*a*]P metabolism and carcinogenic risk by arsenic. This understanding will also help to validate and elucidate on the enhancement of lung cancer among cigarette smokers in arseniasis areas.

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