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Preferential Induction of CYP1A1 and CYP1B1 in CCSP-Positive Cells

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Both benzo[a]pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are potent ligands of aryl hydrocarbon receptors (AhR). Although animal studies indicate that both compounds induce pathological changes in the peripheral lung, the specific cell type involved remains unclear. Clara cells, expressing Clara cell specific protein (CCSP) and abundant in cytochrome P450, are nonciliated bronchiolar epithelial cells in the peripheral lung. Here we explore the hypothesis that CCSP-positive Clara cells are highly responsive to AhR ligands and are the primary cell type involved in BaP- and TCDD-induced toxicities. The responsiveness to AhR ligands was evaluated by measuring the respective mRNA and protein levels of cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) using real-time RT-PCR and immunocytochemistry assays. Two *in vitro* models were used: primary cultures of human small airway epithelial (SAE) cells and rat lung slice cultures. In the presence of calcium, human SAE cells differentiated into CCSP-positive cells. BaP- and TCDD-induced mRNA and protein levels of CYP1A1 and CYP1B1 levels were significantly elevated in CCSP-positive cell cultures. Similarly, AhR mRNA and protein levels were increased in CCSP-positive cell cultures, as determined by real-time RT-PCR and Western blot analysis. When rat lung slice cultures were treated with BaP or TCDD for 24 h, CYP1A1 and CYP1B1 proteins were strongly induced in Clara cells. These results indicate that, in the peripheral lung of both rats and humans, CCSP-positive cells (Clara cells) may be more sensitive to AhR ligands than other cell types.

Key Words: **BaP; TCDD; Clara cells; CYP1A1; CYP1B1; lung.**

Exposure to environmental factors such as dioxins, polycyclic aromatic hydrocarbons (PAH), tobacco smoke, and various air pollutants have been suggested to enhance the risk of chronic obstructive pulmonary diseases (COPD) and lung cancer (Bertazzi *et al*., 2001; Boffetta *et al*., 1997; Kogevinas, 2000). Benzo[a]pyrene (BaP) is the major PAH found in tobacco smoke, which is strongly associated with an increased risk of COPD and lung cancer (Hecht, 1999). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent dioxin. In animal studies, TCDD and BaP both induce pathological changes in the peripheral lungs (Brix *et al*., 2004; Hecht *et al*., 2002; Stoner *et al*., 1984; Tritscher *et al*., 2000). For example, TCDD induces bronchiolar metaplasia in bronchioloalveolar interfaces (Brix *et al*., 2004; Tritscher *et al*., 2000), and BaP-induced lung adenoma was identified in the peripheral lung in a sensitive strain of mice (Hecht *et al*., 2002; Stoner *et al*., 1984). Thus, certain cell types in the peripheral lung are likely to be highly susceptible to BaP and/or TCDD.

Both BaP and TCDD are ligands for the aryl hydrocarbon receptor (AhR). Ligand interaction induces AhR translocation to the nuclei and heterodimerization with the AhR translocator (Arnt), which subsequently transactivates genes of several drug-metabolizing enzymes, such as cytochrome P4501A1 (CYP1A1) and cytochrome P4501B1 (CYP1B1) (Kress and Greenlee, 1997; Whitlock, 1999). There is ample evidence showing that BaP- and TCDD-induced toxic effects are AhR dependent *in vivo* (Fernandez-Salguero *et al*., 1996; Shimizu *et al*., 2000). BaP-induced carcinogenesis is abolished in AhR-deficient mice (Shimizu *et al*., 2000). Therefore, AhR expression and CYP1A1/CYP1B1 induction are important indicators for susceptibility to BaP and TCDD. In human lung tissues, our studies (Lin *et al*., 2003) and others (Saarikoski *et al*., 1998) both show that AhR and CYP1A1 are mainly expressed in bronchiolar epithelial cells of the peripheral lung. Similarly, AhR is expressed in bronchiolar Clara cells and ciliated cells of rats (Tritscher *et al*., 2000). TCDD treatment increased CYP1A1 expression in the cells showing alveolar-bronchiolar metaplasia (Tritscher *et al*., 2000). These data provide evidence that bronchiolar epithelial cells of both humans and rats are responsive to TCDD.

The bronchiolar epithelia consist of various cell types, including basal cells, ciliated cells, and Clara cells. Bronchiolar Clara cells are a specialized type of nonciliated secretory cells, which are believed to be progenitor cells or local stem cells of the bronchiolar epithelium (Otto, 2002). Several lines of

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evidence suggested that bronchiolar Clara cells are considered to be the precursors of human lung adenocarcinoma (AD) (Dermer, 1982; Herrera *et al*., 1983; Linnoila *et al*., 1992; Takezawa *et al*., 2002). Some studies show that Clara cells are rich in cytochrome P450s (Hukkanen *et al*., 2002). Since the majority of AhR ligand-induced toxicities are dependent on CYP1 expression, we hypothesized that Clara cells might be responsive to AhR ligands, such as TCDD and BaP.

To establish the relationship between exposure to AhR ligands and the pathological changes in the peripheral lung, it is essential to evaluate the responsiveness of Clara cells to AhR ligands, such as TCDD and BaP. However, primary cultures of human Clara cells have not previously been established. It has been reported that, while CYP1A1 may be barely detectable, it was nevertheless inducible by AhR ligands (Whitlock, 1999). Recently human small airway epithelial (SAE) cells have become available that can differentiate to express Clara cell secretory protein (CCSP), a specific marker for Clara cells (Jyonouchi *et al*., 1999). Therefore, we compared the levels of induced CYP1A1/CYP1B1 in SAE cell cultures before and after differentiating into CCSP-positive cells. Furthermore, in order to identify the sensitive cell types in the bronchiolar epithelia, we identified the locations of AhR ligand-induced CYP1A1/CYP1B1 proteins in rat lung slice cultures. Harrigan *et al.* (2004) have demonstrated that BaP induced BaP-DNA adducts formation both in rat lung slice cultures and rat lung *in vivo,* suggesting that rat lung slices had comparable biotransformation ability for BaP. Although several studies showed that mouse lungs are highly sensitive to xenobiotics (Simmonds *et al*., 2004; West *et al*., 2001), Lee and Dinsdale (1995) reported that Aroclor 1254 increased CYP1A1 protein in rat lungs, but not in mouse lungs *in vivo*. Therefore, lung slice cultures were prepared from rats, instead of mice, in our present study. Tissue slice cultures are an *in vitro* system, which retain different cell types in an organ. Tissue slices retain the biochemical capacity and the metabolic function of the whole organ, allowing for identification of specific cell types responsive to toxicants. The results generated from this study should allow us to determine if Clara cells are highly responsive to carcinogenic AhR ligands (BaP and TCDD).

MATERIAL AND METHODS

Chemicals. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from ULTRA Scientific (Kingston, RI). Benzo[a]pyrene (BaP) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). TCDD and BaP were dissolved in DMSO, stored in aliquots, and kept at −20°C until used.

Human SAE cell culture and cell differentiation. Human SAE cells (Clonetics,Walkersville, MD) were cultivated in SAE basal medium (Clonetics, Walkersville, MD) and supplemented with growth factors, including bovine pituitary extract, hydrocortisone, recombinant epidermal growth factor, epinephrine, insulin, triiodothyonine, transferrin, gentamicin/amphotericin-B, retinoic acid, and bovine serum albumin (fatty acid free) (Clonetics, Walkersville, MD). SAE cells were plated at a density of 2500 cells/cm^2. The medium was changed every other day. SAE cells were subcultivated when they became confluent. Cells used in these experiments were kept for a maximum of five passages.

Jyonouchi *et al.* (1999) has reported that a high concentration of Ca^{2+} (1 mM $CaCl_2$) induced differentiation and expression of Clara cell secretory protein (CCSP) in SAE cell cultures. SAE cells were seeded on 12- or 24-well plates at a density of 3×10^4 cells/well. When cells became 70% confluent, the medium was changed to one containing 1 mM Ca^{2+} for 72 h. Cell morphology was examined under a light microscope after hematoxylin staining. We found that SAE cells consisted of two cell types. The majority of these cells were small round cells with scanty to adequate cytoplasm, defined as basal cells. Basal cells are thought to be transient amplifying cells, which can terminally differentiate into epithelial cells (Otto, 2002). A few larger cells showed moderate cytoplasm, defined as epithelial cells. In the presence of Ca^{2+}, the cell appearance changed from a monolayer to a stratified or overlapping appearance, similar to epithelial cells lying on the top of basal cells *in vivo* (airway epithelium).

Treatment of human cells. SAE cells were either preincubated with 1 mM calcium or not. After 72 h preincubation, the culture medium was changed and SAE cells were treated with 1 μM BaP, 1 nM TCDD or a01% DMSO (control). Our preliminary study in human lung cell cultures showed that 1 μM of BaP and 1 nM of TCDD are the minimal doses required to significantly increase both CYP1A1 and CYP1B1 expression. After 24-h incubation, cells were harvested for the real-time RT-PCR assay. For immunocytostaining assay, the incubation of 72 h was required.

Immunocytochemistry of human SAE cells. To clarify the characteristics of SAE cells cultivated in Ca^{2+}-supplemented medium, some markers were used, including anti-human CCSP (UP1, 1:1000 dilution), cytokeratin 7 (CK7) (Clone OV-TL 12/30, 1:50 dilution), cytokeratin 14 (CK14) (Clone 34βE12), chromogranin A (Clone DAK-A3), anti-CYP 1B1 (1:3000 dilution, clone WB-1B1, Gentest, Woburn, MA), and anti-CYP1A1 (1:100 dilution, clone H-70, Santa Cruz Biotechnology, Santa Cruz, CA). The majority of the antibodies studied were purchased from DakoCytomation (Glostrup, Denmark). SAE cells on 24-well plates were fixed with cold methanol for 20 min, air dried, and then incubated with adequately diluted antibodies in a moist chamber for 2 h at room temperature. After washing with PBS buffer, an immunoenzymatic reaction was performed using the Universal LSAB2 kit (DakoCytomation, Glostrup, Denmark). Finally, the brown color was developed with 3′-3′ diaminobenzidine (DakoCytomation, Glostrup, Denmark), and Gill's hematoxylin was used for nuclear counterstaining. The immunopositive cells were then counted and the data represented as the percentage of positive cells over total cell numbers.

Quantitative real-time RT-PCR assay. Total cell RNA was prepared using 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 M-MLV Reverse Transcriptase (Promega, Madison, WI). Quantitative PCR was carried out using the ''SYBR® Green'' PCR kit (Qiagen, Hilden, Germany) and analyzed on a ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystem, Foster City, CA). Primers were chosen with the assistance of the computer program Primer Express (Perkin-Elmer Applied Biosystem, Foster City, CA). These primer sequences and optimal concentrations of CCSP, AhR, Arnt, CYP1A1, CYP1B1, and β-actin are shown in Table 1. The PCR reactions consisted of an initial step 2 min at 50°C, a denaturation step for 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and one at 60°C for 1 min. Quantitative values were obtained from the threshold cycle (C_T) 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 β-actin mRNA content. Fold change $= 2^{-\Delta Ct}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$.

Western immunoblot. The cytosolic homogenates of cells were prepared and analyzed by Western immunoblot. Equivalent amounts of 1% Nonidet

TABLE 1
Primer Sequences and Working Concentrations

Primers	Sequences	Working concentration (nm)
β-actin	5′-tcatgaagtgtgacgtggacatc-3′	100
	5′-caggaggagcaatgatcttgatct-3′	
CCSP	Assay ID: Hs00171092[a]	
	5′-acatcacctacgccagtcgc-3′	400
AhR	5′-tctatgccgcttggaaggat-3′	
	5′-gctgctgcctaccctagtctca-3′	200
Arnt	5′-gctgtccgtgtctggaattgt-3′	
	5′-caccatcccccacagcac-3′	200
CYP1A1	5′-acaaagacacaacgcccctt-3′	
	5′-cggctggatttggagaacgta-3′	200
CYP1B1	5′-tgatccaattctgcctgcact-3′	

Note. Abbreviation: CCSP, Clara cell secretory protein; AhR, aryl hydrocarbon receptor; Arnt, Ah-receptor nuclear translocator; CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1.

[a]TagMan® gene expression assay kit of primers and probes (Applied Biosystems).

P-40 or 1% Triton X-100 lysate protein were denatured in SDS buffer (containing 125 mM Tris, 4% SDS, 25% glycerol, 4 mM EDTA, pH 6.8) and separated by 10% SDS–PAGE. Proteins were transferred to PVDF membrane. An AhR antibody (1:1500 dilution, Biomol, Plymouth Meeting, PA) or β-actin antibody (Sigma, Saint Louis, MO) was used. Bands were visualized using an enhanced chemiluminescence kit according to instructions from the manufacturer (Amersham, Buckinghamshire, UK).

Animals. Male Sprague-Dawley rats (150–250 g) 6 weeks old were purchased from the National Laboratory Animal Center (Taiwan, ROC). The animals were housed in standard cages under a 12 h light/dark cycle and received food and water *ad libitum.* All procedures and experiments with animals in this study were approved by the Animal Care and Use Committee at the National Health Research Institute, Taiwan, ROC.

Rat lung slice preparation and incubation. Rats were anesthetized with sodium thiopental (120 mg/kg) via intra-peritoneal injection. The lungs was perfused with Ringer's solution containing 500 U sodium heparin through right pulmonary artery for 5 min to wash the blood away and prevent blood coagulation in small vessels. Then, the lungs were precisely excised and immediately inflated with 1.5% (w/v) low-melting agarose dissolved in culture medium at 37°C. The culture medium was RPMI 1640 medium containing 1 μM insulin, 0.1 mM hydrocortisone-21-hemisuccinate, 5% fetal calf serum, 50 μg/ml streptomycin, 50 IU/ml penicillin, and 2.5 μg fungizone per liter. Subsequently, rat lungs were placed in ice-cold V-7 solution (4°C, pH 7.4) until completely gelled (Fisher *et al.*, 1996). Cylindrical tissue cores (8 mm diameter) were prepared from the lung tissues, which were cut to form 450-μm-thick lung slices using a Vitron tissue slicer (Vitron Inc., Tucson, AZ). Every two to three lung slices were floated on a titanium mesh of Teflon roller insert. These inserts were placed into 20-ml glass scintillation culture vials containing 2 ml of culture medium. Culture vials were capped (the cap has a central 2-mm hole) and placed horizontally into the dynamic organ culture incubator at 37°C, 5% CO_2 and 95% O_2. After 2 h the culture medium was changed, and the lung slices were treated with 10 μM BaP, 1 nM TCDD, or 0.1% DMSO (control) for 24 h. Harrigan *et al.* (2004) demonstrated that BaP-DNA adducts in rat lung slices were not detectable until the dose of BaP was increased to 10 μM. Our preliminary data showed that 10 μM BaP and 1 nM TCDD induced similar levels of *CYP1A1* mRNA *in vitro.* Therefore, these doses were used for lung slices. The lung slices were then fixed with 10% buffered formalin, dehydrated with graded ethanol, and embedded with paraffin for immunohistochemical analysis.

Immunohistochemistry for rat lung slices. The rat lung slices were cut to sections of 4–5 μm thickness on Silane-coated microscope slides (DakoCytomation, Glostrup, Denmark). Paraffin was removed from the sections by xylene, and they were rehydrated through graded ethanol to distilled water. For antigen detection, sections were placed in a microwave oven in a citrate buffer (pH 6) for 20–30 min, cooled for 20 min at room temperature and then washed with distilled water. To block endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide in distilled water for 20 min. Antibodies included anti-CYP1B1, anti-CYP1A1, and anti-rat CCSP (1:4000 dilution, Upstate, Charlottesville, VA) for rat lung slices. These antibodies were incubated overnight at 4°C in a moist chamber, following which an immunoenzymatic reaction was performed using the Universal LSAB2 kit. Finally, the brown color was developed and nuclei stained as for cells (see above).

Statistical analysis. Comparisons between treated and control groups were calculated using the Student's *t*-test.

RESULTS

Ca^{2+}-Induced Differentiation in Human SAE Cell Cultures

CK 7 and CK 14 are specific markers for human respiratory epithelial cells and basal cells, respectively. Respiratory basal cells are thought to differentiate into ciliated and nonciliated (Clara cell) cells, whose CK14 protein is lost (Nakajima *et al.*, 1998; Otto, 2002). In SAE cell cultures, epithelial and basal cells were distinguished by cell morphology as described in Methods. Regardless of Ca^{2+} supplementation, CK7 expression was more common in epithelial cells (24.0% and 39.2%) than in basal cells (1.0% and 1.6%) (Table 2). However, the prevalence of CK14 expression was similar in epithelial and basal cells (Table 2), suggesting both cell types had an ability to differentiate. Chromogranin A and surfactant protein C are markers for neuroendocrine cells and type II pneumocytes, respectively. Neither chromogranin A immunostaining, nor the mRNA level of surfactant protein C, was detectable in SAE cells cultivated in a Ca^{2+}-supplemented medium (data not

TABLE 2
Comparison of the Characteristics of SAE Cells Cultivated in Ca^{2+}-Free or Ca^{2+}-Supplemented Medium

	Ca^{2+}-free		Ca^{2+}-supplemente	
Cell types	CK7[a]	CK14[a]	CK7[a]	CK14[a]
Epithelial cells	24.0 ± 4.4[b]	59.4 ± 7.1	39.2 ± 26.5	78.1 ± 26.9
Basal cells	1.0 ± 0.7	50.8 ± 15.0	1.6 ± 2.0	80.6 ± 17.7[c]

[a]Protein was detected using a immunocytochemical stain in 24-well plates. Protein expression was scored as the percentage of immunostained cells and is represented as the mean ± SD from four replicates.

[b]Compared with basal cells in the same type of medium, $p < 0.05$.

[c]Compared with the same cell type in Ca^{2+}-free medium, $p < 0.05$.

TABLE 3
Expression of CCSP in SAE Cells Cultivated with Ca^{2+}-Free or Supplemented Medium

	Calcium-free		Calcium-supplemented	
Cell types	CCSP mRNA[a]	CCSP protein[b]	CCSP mRNA[a]	CCSP protein[b]
SAE cells	1.5 ± 0.9	0.1 ± 0.3	586.6 ± 176.5[c]	10.3 ± 6.7
Epithelial cells		0.6 ± 1.2		19.2 ± 6.6[c]
Basal cells		0.0 ± 0.0		0.5 ± 0.6

[a]CCSP mRNA molecules relative to 10^8 β-actin molecules was determined using real-time RT-PCR. Each data is the mean of four replicates

[b]CCSP protein was detected by immunocytochemical stain in 24-well plates. CCSP expression is scored as the percentage of immunostained cells as the mean ± SD from four replicates.

[c]Compared with cells in Ca^{2+}-free medium, $p < 0.05$.

shown). This result indicates that there was no neuroendocrine and type II pneumocyte differentiation in these cultures.

CCSP is a specific marker for Clara cells. After Ca^{2+}-supplementation, the CCSP mRNA levels were markedly elevated in SAE cell cultures (Table 3). Utilizing immunocytochemical staining, CCSP immunoreactivity was not detectable in basal cells but detected in 0.6% of epithelial cells in the Ca^{2+}-free condition (Table 3). After Ca^2 -supplementation for 72 h, the prevalence of CCSP immunoreactivity was 0.5% in basal cells and 19.2% in epithelial cells (Table 3). Approximately 80% of epithelial cells were CCSP-negative but CK14-positive. These data suggest that Ca^{2+} induces SAE cell cultures to differentiate into CCSP-positive cell cultures.

Comparison of CYP1A1 and CYP1B1 Induction in SAE and CCSP-Positive Cell Cultures

Several studies have indicated that some cytochrome P450s are rich in Clara cells (Hukkanen *et al*., 2002). Therefore, constitutive and AhR ligand (TCDD and BaP)-induced expression of CYP1A1 and CYP1B1 were examined and compared in SAE (Ca^{2+}-free medium) and CCSP-positive (high Ca^{2+} medium) cell cultures. Regardless of the presence of Ca^{2+}, CYP1A1 mRNA was barely detectable (Figs. 1A and 1C). CYP1B1 mRNA was readily detectable in SAE and CCSP-positive cell cultures (Figs. 1B and 1D). After TCDD treatment, CYP1A1 mRNA levels (4378×10^{-5}) in CCSP-positive cell cultures were much higher than the levels (702×10^{-5}) in SAE cell cultures (Fig. 1A). TCDD-induced CYP1B1 mRNA levels were also increased in CCSP-positive cell cultures (Fig. 1B). Similarly, after BaP treatment, CYP1A1 mRNA levels were 1249 and 99 ($\times 10^{-5}$) in CCSP-positive and SAE cell cultures, respectively (Fig. 1C). BaP-induced CYP1B1 mRNA levels increased approximately two-fold with Ca^{2+} presence (Fig. 1D). Taken together, TCDD- and

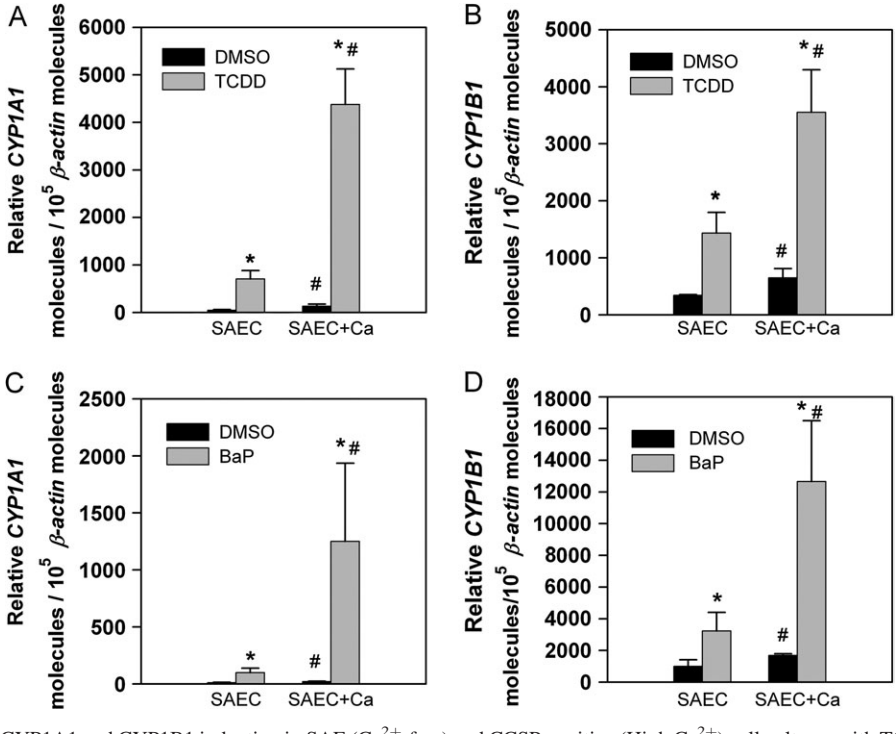

FIG. 1. Comparison of CYP1A1 and CYP1B1 induction in SAE (Ca^{2+}-free) and CCSP-positive (High Ca^{2+}) cell cultures with TCDD or BaP treatment. SAE cells were cultivated in Ca^{2+}-free or supplemented (1 mM) medium for 72 h and were then treated with (A and B) 1 nM TCDD, (C and D) 1 μM BaP or 0.01% DMSO (control solvent) for 24 h. The relative gene expression of (A and C) CYP1A1 and (B and D) CYP1B1 was determined using real-time RT-PCR. ''*'' represents $p < 0.05$ as compared with DMSO-treated cells. ''#'' represents $p < 0.05$ as compared with SAE cell cultures.

TABLE 4
Expression of CYP1A1 and CYP1B1 on CCSP-Positive Cell Cultures Treated with TCDD

	CYP1A1[a]		CYP1B1[a]	
Cell types	DMSO	1 nM TCDD	DMSO	1 nM TCDD
Epithelial cells	0.0 ± 0.0	65.1 ± 7.5[b,c]	22.3 ± 15.8	47.4 ± 16.8[c]
Basal cells	0.0 ± 0.0	10.4 ± 2.9[b]	8.6 ± 10.9	17.1 ± 9.9

[a]Cells were incubated with DMSO or TCDD for 72 h. Protein was detected by immunocytochemical stain in 24-well plates. Expressions were scored as the percentage of immunostained cells as the mean ± SD from four replicates.
[b]Compared with 0.01% DMSO-treated cells, $p < 0.05$.
[c]Compared with basal cells, $p < 0.05$.

BaP-induced CYP1A1 and CYP1B1 levels were much higher in CCSP-positive cell cultures than in SAE cell cultures.

In order to localize the CYP1A1 and CYP1B1 induced proteins in CCSP-positive cell cultures, an immunocytochemical assay was performed. We found that CYP1B1, but not CYP1A1, was detectable in CCSP-positive cell cultures (Table 4). Furthermore, CYP1B1 immunoreactivity was more prevalent in epithelial cells (22.3%) than in basal cells (8.6%) within CCSP-positive cell cultures (Table 4). After TCDD treatment for 72 h, CYP1A1 and CYP1B1 immunoreactivities were increased in both epithelial and basal cells. Similarly, these immunoreactivities were more common in epithelial cells (65.1% and 47.4%) than in basal cells (10.4% and 17.1%) (Table 4). Since CCSP immunoreactivity also located in epithelial cells (Table 3), these results implied that CYP1A1 and CYP1B1 are more inducible by AhR ligands in CCSP-positive epithelial cells than in basal cells.

Increased AhR Expression in CCSP-Positive Cell Cultures

It is well known that AhR and Arnt regulate AhR ligand-induced CYP1A1 and CYP1B1 expression (Kress and Greenlee, 1997; Whitlock, 1999). AhR and Arnt were constitutively expressed in SAE cells (Figs. 2A and 2C). Consistent with increased CYP1A1/CYP1B1 induction, the AhR mRNA levels were significantly increased in CCSP-positive cell cultures (Fig. 2A). The level of AhR also increased two-fold in CCSP-positive cell cultures (Fig. 2B). Using immunocytostaining, AhR expression was observed in both epithelial cells and basal cells. The AhR immunointensity and the percentages of AhR-positive cells were similar between SAE and CCSP-positive cell cultures (data not shown). This inconsistent finding, the result of immunocytostaining assay compared with the results of RT-PCR assay and Western immunoblot, may be due to the limited sensitivity of immunocytochemistry. Levels of Arnt mRNA were only slightly increased in CCSP-positive cells (Fig. 2C). These results imply that increased AhR expression might partially contribute to the elevated induction of CYP1A1 and CYP1B1 in CCSP-positive cell cultures.

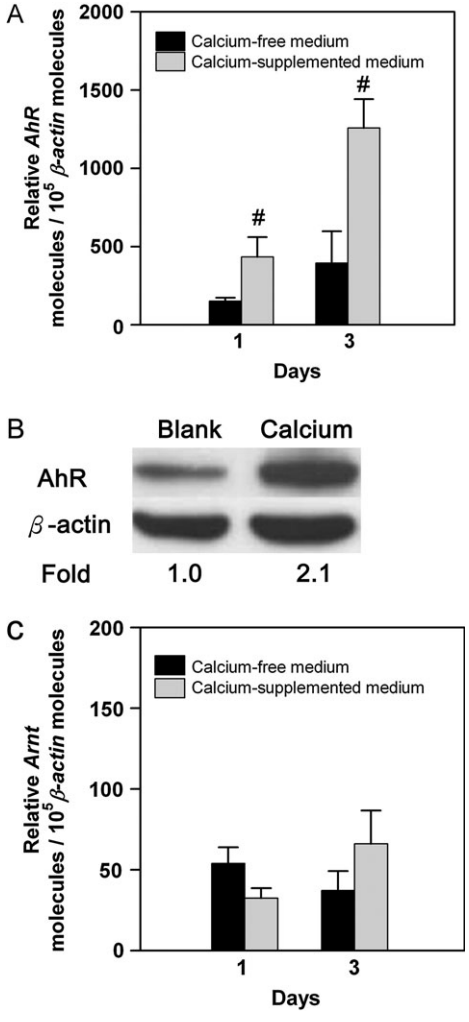

FIG. 2. Effect of Ca^{2+} on the expression of AhR and Arnt in SAE cell cultures. SAE cells were cultivated in Ca^{2+}-free or supplemented medium for 72 h. Total cell RNA and protein were then extracted. Relative gene expression of (A) AhR and (C) Arnt were determined by real-time RT-PCR. (B) The expression of AhR relative to β-actin was quantified using Western immunoblotting. "#" represents $p < 0.05$ as compared with SAE cells cultivated in Ca^{2+}-free medium.

Colocalization of CCSP, CYP1A1, and CYP1B1 Proteins in Rat Lung Slices

In CCSP-positive cell cultures, CYP1A1 and CYP1B1 expression were highly inducible. We further used an *in vitro* model of rat lung slice cultures to investigate the locations of TCDD- and BaP-induced effects. In rat lung slices, CCSP-positive cells were nonciliated epithelial cells, namely Clara cells (Figs. 3A, 3D, and 3G). Rat Clara cells were found in the bronchi and bronchioles, but not in the alveoli. Clara cells were more common in the bronchioles than in the bronchi (52% vs. 35%). After treatments with TCDD and BaP, increased CYP1A1 immunoreactivity was detected in the cytoplasm of nonciliated and ciliated bronchiolar epithelial cells (Figs. 3E

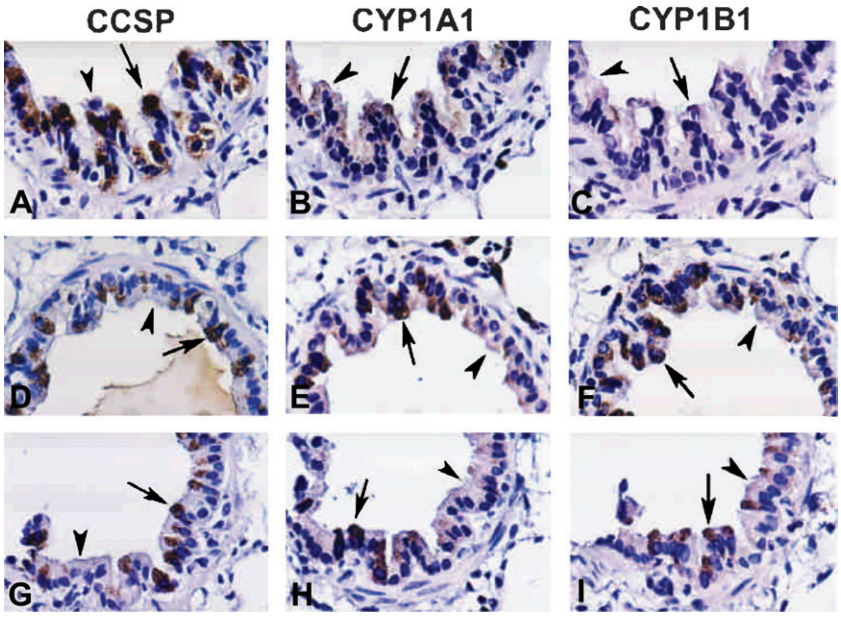

FIG. 3. Colocalization of Clara cell secretory protein (CCSP) and CYP1A1/CYP1B1 in rat lung slice cultures. Immunohistochemistry, ×400. Rat lung slices were cultivated in medium containing (A to C) 0.1% DMSO, (D to F) 1 nM TCDD, and (G to I) 10 μM BaP for 24 h. Rat lung slices were then fixed and paraffin-embedded. Serial sections were stained with (A, D, and G) anti-CCSP, (D, E, and H) anti-CYP1A1, and (C, F, and I) anti-CYP1B1. Positive immunoreactivity exhibited a cytoplasmic brown coloration. Bronchiolar Clara cells (arrow) were nonciliated cells those partly coexpressed CYP1A1/CYP1B1. Ciliated cells (arrowhead) were CCSP-negative, but CYP1A1/CYP1B1 weakly positive compared with Clara cells.

and 3H), in comparison with DMSO-treated lung slices (Fig. 3B). It should be noted that nonciliated cells (Clara cells) showed a much stronger intensity than ciliated cells (Figs. 3E and 3H). CYP1B1 immunoreactivity was weak but constitutively observed in the smooth muscles of the vascular and bronchial walls (data not shown). The locations for BaP and TCDD-induced immunoreactions of CYP1B1 were similar to those of CYP1A1 in rat lung slices (Figs. 3C, 3F, and 3I). Thus, these results provide evidence that CYP1A1/CYP1B1 induction is more marked in Clara cells than in other bronchiolar epithelial cells of rat lung slices.

DISCUSSION

The objective of this study was to determine whether human Clara cells or CCSP-positive cells are responsive to AhR ligands. In the primary cultures of human SAE (basal) cells, AhR ligands (TCDD and BaP)-induced CYP1A1/1B1 expression was highly elevated when cells differentiated into CCSP-positive cells, indicating human CCSP-positive cells are highly responsive to AhR ligands. However, in the Ca^{2+}-induced SAE differentiating system, human CCSP-positive cells retained the ability to differentiate (CK14 positive) and would be considered as stem cells or progenitor cells of the bronchiolar epithelia. To understand if terminally differentiated Clara cells are responsive to AhR ligands, we identified the location of TCDD/BaP-induced CYP1A1/1B1 proteins in the rat lung slice cultures. It was found that CYP1A1/1B1 proteins were consistently located in Clara cells. These results indicate that the responsiveness to AhR ligands was highly increased when SAE (basal) cells had differentiated into CCSP-positive or Clara cells. In the other words, this finding implies that Clara cells in the peripheral lung of humans and rats were more sensitive to AhR ligands than basal and other bronchiolar epithelial cells. Type II pneumocytes have been demonstrated to be susceptible to toxicants (Monteil *et al*., 1999; Tatrai *et al*., 2001). However, type II pneumocytes were not present in our present systems. Thus we cannot ignore the possibility of CYP1A1 and CYP1B1 induction in type II pneumocytes.

The distribution of Clara cells is similar in rats and humans, being more prevalent in the peripheral than in the central airways (Boers *et al*., 1999). Clara cells are believed to contribute to cell renewal in the peripheral airway epithelium (Aliotta *et al*., 2005; Hong *et al*., 2001; Otto, 2002). In addition to being progenitor cells for replacing injured epithelium, Clara cells are known to synthesize and secrete CCSP as well as protease (Massaro *et al*., 1994; Singh and Katyal, 1997). CCSP inhibits phospholipase A2, which is involved in the regulation of the inflammatory process (Anderson *et al*., 1994; Johnston *et al*., 1997). Protease secreted by Clara cells is trypsin-like and can inhibit leukocyte protease activity to maintain the protease-antiprotease balance in the lung (Massaro *et al*., 1994). TCDD and BaP have been shown to induce cell apoptosis or inhibit cell growth in an AhR dependent manner (Lin *et al*., 2004; Solhaug *et al*., 2005). In the other words, CYP1A1/1B1

induction is required for TCDD and BaP-induced cytotoxicity. This implication is supported by some *in vivo* studies. For example, it was reported that the number of Clara cells is reduced in the lungs of cigarette smokers (Lumsden *et al*., 1984; Shijubo *et al*., 1997). In rats, TCDD exposure caused pathological changes in the bronchiolar epithelia, primarily in Clara cells (Brix *et al*., 2004; Tritscher *et al*., 2000). Therefore, AhR ligands may interact with Clara cells to disturb the cell renewing function.

Previously we showed that TCDD inhibited cell growth, but failed to induce cell death, in human bronchial epithelial cells (Lin *et al*., 2004). In our present study, CCSP-positive cell cultures were the mixtures of basal cells, CCSP-positive epithelial cells and CCSP-negative epithelial cells. Furthermore, in the presence of calcium, epithelial cells were consistently differentiated from basal cells. Therefore, we were unable to selectively evaluate effects of TCDD and BaP on growth of CCSP-positive cells. In lung slice cultures, we failed to observe morphological change in TCDD and BaP-treated slices 24 h later. It is likely that the incubation time (24 h) was too short to cause cytotoxicity in slice cultures.

Using light microscopy, electron microscopy, and immunohistochemical methods, earlier studies suggested that bronchiolar Clara cells constitute one of the precursor cells for peripheral lung AD (Dermer, 1982; Kitamura *et al.*, 1997; Mori *et al.*, 1998). However, several studies showed that CCSP expression is reduced during the development of lung carcinogenesis. CCSP expression is widely observed in normal bronchiolar epithelia, but less often in human atypical bronchiolar lesions and AD (Jensen *et al.*, 1994; Nomori *et al.*, 1994). Bernard *et al.* (1992) showed that CCSP levels are reduced in serum and bronchoalvolar lavage fluids obtained from lung cancer patients. The loss of CCSP protein also occurred in a transgenic mouse model for the transformation of Clara cells to lung AD (Hicks *et al.*, 2003). These data imply that Clara cells were either damaged or transformed into tumor cells during lung carcinogenesis. Since CCSP-positive cells are highly responsive to carcinogenic AhR ligands, the relationship between exposure of AhR ligands and the development of lung AD deserves further investigation.

One of our major findings was that CYP1A1/1B1 induction and AhR expression were increased during Clara cell differentiation. Similarly, CYP1A1 induction and AhR expression were also increased when proliferating keratinocytes were induced to differentiate (Wanner *et al*., 1995). It appeared that increased AhR expression at least partially explains the increased CYP1A1/1B1 induction in more differentiated keratinocytes and CCSP-positive lung cells (Wanner *et al*., 1995). By contrast, AhR expression was decreased during adipose differentiation (Shimba *et al*., 2001). These findings suggest that the relationship between AhR expression and differentiation is cell-type specific. Furthermore, the causal relationship between AhR expression and differentiation is not yet established and deserves further investigation.

Although CYP1A1 protein was detected in Clara cells from rats treated with AhR ligands (PCB or 3-methylcholanthrene) *in vivo* (Lee and Dinsdale, 1995), CYP1A1 induction by AhR ligands was not observed in primary cultures of rat lung cells (Solhaug *et al*., 2004, 2005). It is likely that CYP1A1 inducibility in normal lung cells was lost in the *in vitro* culture system. CYP1A1 and CYP1B1 are involved in the conversion of BaP into an ultimate metabolite, which forms DNA adducts (Kim *et al*., 1998; Shimada *et al*., 1999). Recently, Harrigan *et al.* (2004) demonstrated that *in vitro* incubation with BaP increased BaP-DNA adduct levels in rat lung slices, suggesting that drug metabolizing enzymes were induced by BaP in lung slice cultures. In our present study, we successfully demonstrated TCDD/BaP-induced CYP1A1/1B1 proteins in Clara cells of rat lung slice cultures, which have been maintained *in vitro* for 3 days. Our results not only prove that Clara cells are highly responsive to AhR ligands, but also indicate that rat lung slice cultures are an excellent *in vitro* model for studying toxicant metabolism in the lung.

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REFERENCES

Aliotta, J. M., Passero, M., Meharg, J., Klinger, J., Dooner, M. S., Pimentel, J., and Quesenberry, P. J. (2005). Stem cells and pulmonary metamorphosis: New concepts in repair and regeneration. *J. Cell. Physiol.* **204,** 725–741.

Anderson, O., Noack, G., Robertson, B., Glaumann, H., Sonnenfeld, T., and Lund, J. (1994). Ontogeny of a human polychlorinated biphenyl-binding protein. Level of expression in tracheal aspirates in bronchopulmonary dysplasia. *Chest* **105,** 17–22.

Bernard, A., Marchandise, F. X., Depelchin, S., Lauwerys, R., and Sibille, Y. (1992). Clara cell protein in serum and bronchoalveolar lavage. *Eur. Respir. J* **5,** 1231–1238.

Bertazzi, P. A., Consonni, D., Bachetti, S., Rubagotti, M., Baccarelli, A., Zocchetti, C., and Pesatori, A. C. (2001). Health effects of dioxin exposure: A 20-year mortality study. *Am. J. Epidemiol.* **153,** 1031–1044.

Boers, J. E., Ambergen, A. W., and Thunnissen, F. B. (1999). Number and proliferation of clara cells in normal human airway epithelium. *Am. J. Respir. Crit. Care Med.* **159,** 1585–1591.

Boffetta, P., Jourenkova, N., and Gustavsson, P. (1997). Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. *Cancer Causes Control* **8,** 444–472.

Brix, A. E., Jokinen, M. P., Walker, N. J., Sells, D. M., and Nyska, A. (2004). Characterization of bronchiolar metaplasia of the alveolar epithelium in female Sprague-Dawley rats exposed to 3,3′,4,4′,5-pentachlorobiphenyl (PCB126). *Toxicol. Pathol.* **32,** 333–337.

Dermer, G. B. (1982). Origin of bronchioloalveolar carcinoma and peripheral bronchial adenocarcinoma. *Cancer* **49,** 881–887.

Fernandez-Salguero, P. M., Hilbert, D. M., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1996). Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. *Toxicol. Appl. Pharmacol.* **140,** 173–179.

Fisher, R. L., Hasal, S. J., Sanuik, J. T., Hasal, K. S., Gandolfi, A. J., and Brendel, K. (1996). Cold- and cryopreservation of dog liver and kidney slices. *Cryobiology* **33,** 163–171.

Harrigan, J. A., Vezina, C. M., McGarrigle, B. P., Ersing, N., Box, H. C., Maccubbin, A. E., and Olson, J. R. (2004). DNA adduct formation in precision-cut rat liver and lung slices exposed to benzo[a]pyrene. *Toxicol. Sci.* **77,** 307–314.

Hecht, S. S. (1999). Tobacco smoke carcinogens and lung cancer. *J. Natl. Cancer Inst.* **91,** 1194–1210.

Hecht, S. S., Kenney, P. M., Wang, M., and Upadhyaya, P. (2002). Benzyl isothiocyanate: An effective inhibitor of polycyclic aromatic hydrocarbon tumorigenesis in A/J mouse lung. *Cancer Lett.* **187,** 87–94.

Herrera, G. A., Alexander, C. B., and DeMoraes, H. P. (1983). Ultrastructural subtypes of pulmonary adenocarcinoma. A correlation with patient survival. *Chest* **84,** 581–586.

Hicks, S. M., Vassallo, J. D., Dieter, M. Z., Lewis, C. L., Whiteley, L. O., Fix, A. S., and Lehman-McKeeman, L. D. (2003). Immunohistochemical analysis of Clara cell secretory protein expression in a transgenic model of mouse lung carcinogenesis. *Toxicology* **187,** 217–228.

Hong, K. U., Reynolds, S. D., Giangreco, A., Hurley, C. M., and Stripp, B. R. (2001). Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *Am. J. Respir. Cell Mol. Biol.* **24,** 671–681.

Hukkanen, J., Pelkonen, O., Hakkola, J., and Raunio, H. (2002). Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. *Crit. Rev. Toxicol.* **32,** 391–411.

Jensen, S. M., Jones, J. E., Pass, H., Steinberg, S. M., and Linnoila, R. I. (1994). Clara cell 10 kDa protein mRNA in normal and atypical regions of human respiratory epithelium. *Int. J. Cancer* **58,** 629–637.

Johnston, C. J., Mango, G. W., Finkelstein, J. N., and Stripp, B. R. (1997). Altered pulmonary response to hyperoxia in Clara cell secretory protein deficient mice. *Am. J. Respir. Cell Mol. Biol.* **17,** 147–155.

Jyonouchi, H., Sun, S., Iijima, K., Wang, M., and Hecht, S. S. (1999). Effects of anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene on human small airway epithelial cells and the protective effects of myo-inositol. *Carcinogenesis* **20,** 139–145.

Kim, J. H., Stansbury, K. H., Walker, N. J., Trush, M. A., Strickland, P. T., and Sutter, T. R. (1998). Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1. *Carcinogenesis* **19,** 1847–1853.

Kitamura, H., Kameda, Y., Ito, T., Hayashi, H., Nakamura, N., Nakatani, Y., Inayama, Y., and Kanisawa, M. (1997). Cytodifferentiation of atypical adenomatous hyperplasia and bronchioloalveolar lung carcinoma: Immunohistochemical and ultrastructural studies. *Virchows Arch.* **431,** 415–424.

Kogevinas, M. (2000). Studies of cancer in humans. *Food Addit. Contam.* **17,** 317–324.

Kress, S., and Greenlee, W. F. (1997). Cell-specific regulation of human CYP1A1 and CYP1B1 genes. *Cancer Res.* **57,** 1264–1269.

Lee, M. J., and Dinsdale, D. (1995). The subcellular distribution of NADPH-cytochrome P450 reductase and isoenzymes of cytochrome P450 in the lungs of rats and mice. *Biochem. Pharmacol.* **49,** 1387–1394.

Lin, P., Chang, Y. C., Chen, C. H., Yang, W. J., Cheng, Y. H., and Chang, L. W. (2004). A comparative study on the effects of 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin polychlorinated biphenyl126 and estrogen in human bronchial epithelial cells. *Toxicol. Appl. Pharmacol.* **195,** 83–91.

Lin, P., Chang, H., Ho, W. L., Wu, M. H., and Su, J. M. (2003). Association of aryl hydrocarbon receptor and cytochrome P4501B1 expressions in human non-small cell lung cancers. *Lung Cancer* **42,** 255–261.

Linnoila, R. I., Jensen, S. M., Steinberg, S. M., Mulshine, J. L., Eggleston, J. C., and Gazdar, A. F. (1992). Peripheral airway cell marker expression in non-small cell lung carcinoma. Association with distinct clinicopathologic features. *Am. J. Clin. Pathol.* **97,** 233–243.

Lumsden, A. B., McLean, A., and Lamb, D. (1984). Goblet and Clara cells of human distal airways: Evidence for smoking induced changes in their numbers. *Thorax* **39,** 844–849.

Massaro, G. D., Singh, G., Mason, R., Plopper, C. G., Malkinson, A. M., and Gail, D. B. (1994). Biology of the Clara cell. *Am. J. Physiol.* **266,** L101–L106.

Monteil, C., Le Prieur, E., Buisson, S., Morin, J. P., Guerbet, M., and Jouany, J. M. (1999). Acrolein toxicity: Comparative *in vitro* study with lung slices and pneumocytes type II cell line from rats. *Toxicology* **133,** 129–138.

Mori, M., Kaji, M., Tezuka, F., and Takahashi, T. (1998). Comparative ultrastructural study of atypical adenomatous hyperplasia and adenocarcinoma of the human lung. *Ultrastruct. Pathol.* **22,** 459–466.

Nakajima, M., Kawanami, O., Jin, E., Ghazizadeh, M., Honda, M., Asano, G., Horiba, K., and Ferrans, V. J. (1998). Immunohistochemical and ultrastructural studies of basal cells, Clara cells and bronchiolar cuboidal cells in normal human airways. *Pathol. Int.* **48,** 944–953.

Nomori, H., Morinaga, S., Kobayashi, R., and Torikata, C. (1994). Protein 1 and Clara cell 10-kDa protein distribution in normal and neoplastic tissues with emphasis on the respiratory system. *Virchows Arch.* **424,** 517–523.

Otto, W. R. (2002). Lung epithelial stem cells. *J. Pathol.* **197,** 527–535.

Saarikoski, S. T., Husgafvel-Pursiainen, K., Hirvonen, A., Vainio, H., Gonzalez, F. J., and Anttila, S. (1998). Localization of CYP1A1 mRNA in human lung by *in situ* hybridization: Comparison with immunohistochemical findings. *Int. J. Cancer* **77,** 33–39.

Shijubo, N., Itoh, Y., Yamaguchi, T., Shibuya, Y., Morita, Y., Hirasawa, M., Okutani, R., Kawai, T., and Abe, S. (1997). Serum and BAL Clara cell 10 kDa protein (CC10) levels and CC10-positive bronchiolar cells are decreased in smokers. *Eur. Respir. J.* **10,** 1108–1114.

Shimada, T., Gillam, E. M., Oda, Y., Tsumura, F., Sutter, T. R., Guengerich, F. P., and Inoue, K. (1999). Metabolism of benzo[a]pyrene to trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene by recombinant human cytochrome P450 1B1 and purified liver epoxide hydrolase. *Chem. Res. Toxicol.* **12,** 623–629.

Shimba, S., Wada, T., and Tezuka, M. (2001). Arylhydrocarbon receptor (AhR) is involved in negative regulation of adipose differentiation in 3T3-L1 cells: AhR inhibits adipose differentiation independently of dioxin. *J. Cell Sci.* **114,** 2809–2817.

Shimizu, Y., Nakatsuru, Y., Ichinose, M., Takahashi, Y., Kume, H., Mimura, J., Fujii-Kuriyama, Y., and Ishikawa, T. (2000). Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 779–782.

Simmonds, A. C., Ghanayem, B. I., Sharma, A., Reilly, C. A., Millen, B., Yost, G. S., and Forkert, P. G. (2004). Bioactivation of 1,1-dichloroethylene by CYP2E1 and CYP2F2 in murine lung. *J. Pharmacol. Exp. Ther.* **310,** 855–864.

Singh, G., and Katyal, S. L. (1997). Clara cells and Clara cell 10 kD protein (CC10). *Am. J. Respir. Cell Mol. Biol.* **17,** 141–143.

Solhaug, A., Ovrebo, S., Mollerup, S., Lag, M., Schwarze, P. E., Nesnow, S., and Holme, J. A. (2005). Role of cell signaling in B[a]P-induced apoptosis: Characterization of unspecific effects of cell signaling inhibitors and apoptotic effects of B[a]P metabolites. *Chem. Biol. Interact.* **151,** 101–119.

Solhaug, A., Refsnes, M., Lag, M., Schwarze, P. E., Husoy, T., and Holme, J. A. (2004). Polycyclic aromatic hydrocarbons induce both apoptotic and anti-apoptotic signals in Hepa1c1c7 cells. *Carcinogenesis* **25,** 809–819.

Stoner, G. D., Greisiger, E. A., Schut, H. A., Pereira, M. A., Loeb, T. R., Klaunig, J. E., and Branstetter, D. G. (1984). A comparison of the lung adenoma response in strain A/J mice after intraperitoneal and oral administration of carcinogens. *Toxicol. Appl. Pharmacol.* **72,** 313–323.

Takezawa, C., Takahashi, H., Fujishima, T., Shiratori, M., Morita, Y., Sano, H., Kuroki, Y., and Abe, S. (2002). Assessment of differentiation in adenocarcinoma cells from pleural effusion by peripheral airway cell markers and their diagnostic values. *Lung Cancer* **38,** 273–281.

Tatrai, E., Kovacikova, Z., Karacsony, G., Hudak, A., Adamis, Z., and Ungvary, G. (2001). Effects of sodium diethyldithiocarbamate on type II pulmonary epithelial cells *in vitro*. *J. Toxicol. Environ. Health A* **62,** 207–216.

Tritscher, A. M., Mahler, J., Portier, C. J., Lucier, G. W., and Walker, N. J. (2000). Induction of lung lesions in female rats following chronic exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Pathol.* **28,** 761–769.

Wanner, R., Brommer, S., Czarnetzki, B. M., and Rosenbach, T. (1995). The differentiation-related upregulation of aryl hydrocarbon receptor transcript levels is suppressed by retinoic acid. *Biochem. Biophys. Res. Commun.* **209,** 706–711.

West, J. A., Pakehham, G., Morin, D., Fleschner, C. A., Buckpitt, A. R., and Plopper, C. G. (2001). Inhaled naphthalene causes dose dependent Clara cell cytotoxicity in mice but not in rats. *Toxicol. Appl. Pharmacol.* **173,** 114–119.

Whitlock, J. P., Jr. (1999). Induction of cytochrome P4501A1. *Annu. Rev. Pharmacol. Toxicol.* **39,** 103–125.