The aryl hydrocarbon receptor is a regulator of cigarette smoke induction of the cyclooxygenase and prostaglandin pathways in human lung fibroblasts

C. A. Martey,† C. J. Baglole,‡,§ T. A. Gasiewicz,‡ P. J. Sime,¶ and R. P. Phipps†

†Department of Environmental Medicine, ‡Lung Biology and Disease Program, ‡Division of Pulmonary and Critical Care Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York

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Martey, C. A., C. J. Baglole, T. A. Gasiewicz, P. J. Sime, and R. P. Phipps. The aryl hydrocarbon receptor is a regulator of cigarette smoke induction of the cyclooxygenase and prostaglandin pathways in human lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 289: L391–L399, 2005. First published April 29, 2005; doi:10.1152/ajplung.00062.2005.—Cigarette smoking can lead to chronic lung inflammation and lung cancer. Chronic inflammation, associated with expression of cyclooxygenase-2 (COX-2) and prostaglandins, predisposes to malignancy. We recently demonstrated that human lung fibroblasts are activated by cigarette smoke to express COX-2 and prostaglandin E2 (PGE2). Little is known about the mechanism whereby smoke activates human lung fibroblasts to produce proinflammatory mediators. Herein, we report the central role of the aryl hydrocarbon receptor (AHR) in cigarette smoke extract- (CSE)-induced COX-2, microsomal PGE2 synthase (mPGES), and PGE2 production in human lung fibroblasts. Western blot analysis revealed that primary strains of human lung fibroblasts express AHR and aryl hydrocarbon nuclear translocator protein, supporting the possibility that smoke activates lung fibroblasts through this pathway. Experiments were subsequently performed to determine whether the AHR was activated by CSE. Immunocytochemistry and EMSA analysis revealed that CSE induced nuclear translocation of the AHR in human lung fibroblasts. CSE decreased protein levels of the AHR, consistent with AHR ligand-induced proteasome-mediated degradation. CSE also induced mPGES-1 and COX-2 protein and increased PGE2 production. Treatment of human fibroblasts with AHR antagonists in the presence of CSE inhibited AHR nuclear translocation as well as COX-2, mPGES-1, and PGE2 production. These data indicate that the AHR pathway plays an important role in cigarette smoke-mediated COX-2 and PG production in human lung fibroblasts and may contribute to tobacco-associated inflammation and lung disease.

Cigarette smoke is a major risk factor for several lung diseases, including chronic obstructive pulmonary disease (COPD), asthma, and lung cancer. Lung cancer is the leading cause of cancer mortality in the United States (3), and the most prominent factor for developing COPD is tobacco smoke. There are 12.1 million patients with COPD in the USA (38). A common feature in the pathogenesis of cigarette smoke-associated lung diseases is inflammation (2). There is now strong evidence that chronic inflammation is linked with an increased risk of developing certain cancers (39, 55). One of the hallmarks of inflammation is increased production of prostaglandins (PG), such as prostaglandin E2 (PGE2), through the induction of the microsomal PGE2 synthases (mPGES) and cyclooxygenases (COX). mPGES-1 and COX-2 are absent in most tissues under normal conditions but are rapidly upregulated during an inflammatory response (36). In contrast, COX-1 and mPGES-2 are constitutively expressed (37, 47, 54). Many types of malignancies have elevated levels of COX-2, including non-small cell lung cancers and premalignant bronchial and alveolar lesions (6, 19, 29, 30). Both cigarette smoke condensate and components of cigarette smoke such as benzo[a]pyrene can induce the expression of COX-2 (2, 32, 63).

We hypothesized that long-term exposure to cigarette smoke, resulting in COX-2 induction and chronic inflammation and cancer, might be attributable to activation of the aryl hydrocarbon receptor (AHR). The AHR is a ligand-activated member of the basic helix-loop-helix Period (Per)/Arnt/Sim family of transcription factors that includes Per, AHR nuclear translocator (Arnt), and hypoxia-inducible factor-1β (35). The AHR mediates the biological and toxic responses of a class of environmental pollutants (e.g., dioxins). Unoccupied AHR is found in the cytoplasm complexed with heat shock protein 90 and other proteins (43). Once an agonist such as the man-made toxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or other aromatic hydrocarbons binds to the AHR, the ligand-AHR complex translocates to the nucleus where it forms a heterodimer with the Arnt. This complex binds to specific DNA sequences called dioxin response elements (DRE) that provide regulatory control over many AHR-responsive genes (43), including members of the cytochrome P-450s and COX-2. COX-2 has a DRE site in its promoter region (28, 61), and polyaromatic hydrocarbons such as TCDD induce COX-2 and PG synthesis (44, 61).

We have recently shown that COX-2, mPGES, and PGE2 are upregulated in normal human lung fibroblasts after exposure to cigarette smoke extract (CSE) (32) but not in A549 human alveolar or 16HBE human bronchial epithelial cells (data not shown). Fibroblasts are considered to be a major cell type that expresses COX-2 and synthesizes PG in humans (53, 65). Fibroblasts act as tissue sentinel cells and orchestrate and incite lung inflammation via their ability to become activated by lung irritants, including cigarette smoke. Unlike epithelial cells, fibroblasts have received relatively little attention as a target of tobacco smoke. This is despite the fact that fibroblasts are key inciters and orchestrators of chronic inflammation, are abundant structural cells (24, 57), and are thought to be targets of...

* C. A. Martey and C. J. Baglole contributed equally to this work.

Address for reprint requests and other correspondence: R. P. Phipps, Univ. of Rochester School of Medicine and Dentistry, Dept. of Environmental Medicine, 601 Elmwood Ave., Box 850, Rochester, NY 14642 (e-mail: Richard_Phipps@urmc.rochester.edu).

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the water-soluble components of cigarette smoke (21). We hypothesize that CSE induces COX-2 and PG production in normal human lung fibroblasts through activation of the AHR pathway. Herein we describe the molecular mechanisms whereby smoke activates human fibroblasts, and we identify the AHR pathway as a potential therapeutic target to modulate inflammation and tumorigenesis.

MATERIALS AND METHODS

Cell cultures. Primary human lung fibroblast strains (each from a different human being) were established as previously described (10). These cells were identified as fibroblasts by their morphology, adherent nature, expression of vimentin and types I and III collagen, and lack of expression of cytokeratin, α-smooth muscle actin, factor VIII, and CD45. Cells were cultured in MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (HyClone Laboratories, Logan, UT) and 50 U/ml gentamicin (Life Technologies) and incubated in humidified 5% CO2/95% air at 37°C as previously described (52). Fibroblasts were used at as early passage as possible and seeded at densities based on the technique used.

Preparation of aqueous CSE. Research grade cigarettes (1R3F) with a filter from the Kentucky Tobacco Research Council were smoked to 0.5 cm above the filter using a modification of the method developed by Carp and Janoff (5, 32). 100% CSE was prepared by bubbling smoke from two cigarettes into 20 ml of serum-free MEM at a rate of 1 cigarette/min. The pH of the MEM was adjusted to 7.4, and the media were sterile filtered with a 0.45-μm filter (25-mm Acrodisc; Pall, Ann Arbor, MI). The CSE (100%) was stored in sterile Eppendorf tubes at 4°C for up to 3 days without any change in potency. Control media were prepared by bubbling air through 20 ml of serum-free MEM with pH adjusted to 7.4 and sterile filtering as described.

RNAse protection assays. Fibroblasts were grown to 70% confluency in six-well plates (seeded ~1 × 10^5 cells/well), after which they were serum starved for 48 h to reduce basal levels of COX-2 and PG. Cells were then treated with the AHR antagonist 3'-methoxy-4'-nitroflavone (MNF) (11, 31) for 1 h followed by cotreatment with 1% CSE as an additional 4 h. Total RNA was isolated from the cells using Tri-Reagent according to the manufacturer’s protocol (Molecular Research Center, Cincinnati, OH), and RNA quantitation was done using spectrophotometry. Probes for COX-1, COX-2, and β-actin (BD Pharmingen, San Diego, CA) were used to run this assay using the manufacturer’s instructions. 32P-labeled riboprobes were synthesized to determine the effects of AHR inhibition on PGE2 levels, cells were serum starved for 48 h before being stimulated with 1% CSE for 24 h. To determine the level of nonspecific staining, cells were incubated under the same conditions with the mouse IgG1 isotype antibody (diluted 1:2 in PBS/BSA). Biotinylated anti-mouse IgG antibody was used for secondary binding (1:200) and incubated for 1 h at room temperature before being incubated with streptavidin-horseradish peroxidase. Antibody binding was visualized using the substrate aminoethylcarbazol (Zymed, South San Francisco, CA).

EMSA. Lung fibroblast strains were grown to confluent monolayers in 10-cm plates (~6 × 10^5 cells/plate) and serum starved for 48 h before being stimulated with 1% CSE for 3 h as shown in Fig. 2C. Control treatments were with serum-free MEM only. Nuclear extracts were prepared as previously described (48). Briefly, cells were washed twice with ice-cold PBS, scraped from the culture dishes, and centrifuged (1,000 × g, 10 min). The cell pellets were resuspended in 400 μl of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 0.5 mM PMFS, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 2 μg/ml aprotinin, 1 mM DTT, and 10 mM sodium molybdate) and allowed to swell on ice for 15 min. Nonidet P-40 was then added to a final concentration of 0.5%. After rapid mixing for 15 s, the nuclei were sedimented (4,000 × g, 1 min), and the supernatants containing cytosolic proteins were collected. The remaining pellet was resuspended in 50 μl of nuclear extract buffer B (hypotonic buffer A supplemented with 20% glycerol and 0.4 M KCl) and shaken vigorously at 4°C for 30 min. Nuclear extracts were obtained from the supernatants after centrifugation (12,000 × g, 15 min) and stored at −80°C. For gel shift assays, 5 μg of each nuclear extract were incubated with 1 ng (~50,000 counts (μl) of 32P-labeled AHR probe (GGAGTTGCGTGAGAAGACCC) and GGCTCTTCTACGCAACTCC; synthesized by Tufts Univ., Boston, MA) at room temperature for 15 min in a reaction mix containing 10 μg of BSA and 1 μl of poly(dI-dC) in binding buffer (7.5% glycerol, 1 mM MgCl2, 0.05 mM EDTA, 0.5 mM DTT, 35 mM NaCl, and 7.5 mM HEPEs, pH 8) in a total volume of 25 μl (32). The DNA/protein complex was separated on 4% native polyacrylamide gels. Nuclear extracts (15 μg) prepared from fibroblasts exposed to CSE for 60 min were used to assay binding specificity. Here, nuclear
extracts were preincubated either with excess unlabeled probe (100×, 15 min) for cold competition or with the AHR antibody (1 μg, 30 min) for supershift analysis before their addition to the reaction mixture. Radioactive bands were detected by autoradiography using Kodak XOMAT MR or MS film with an average exposure time of 18–24 h.

Statistical analysis. Statistical significance was determined using Student’s paired two-tailed t-test where P < 0.05 indicates statistical significance between tested samples. All experiments were repeated three to five times.

RESULTS

Normal human lung fibroblasts express AHR and Arnt protein. To determine whether the AHR pathway may play a role in COX-2 induction and PG production in normal human lung fibroblasts, Western blotting was performed on several human lung fibroblast strains to first establish whether or not AHR and Arnt were expressed (Fig. 1). Whole cell extracts probed with an anti-AHR antibody indicated that this protein was present in all strains tested. Upon activation, the AHR forms a heterodimer with Arnt before DNA binding (34). Western blot analysis on the same cell extracts indicates that, similar to the AHR, Arnt was present in all fibroblast strains tested (Fig. 1).

CSE activates the AHR in normal human lung fibroblasts. Nothing is known about the function of the AHR in human lung fibroblasts. Therefore, to begin to investigate the role of the AHR in CSE-induced fibroblast activation, we first tested the ability of CSE to activate the AHR in vitro. We first examined AHR protein levels following CSE exposure. This was done as the AHR underwent ubiquitination and proteosome-mediated degradation following ligand binding and nuclear localization in nonfibroblastic cells (13, 43, 46). Western blot analysis revealed that exposure of human lung fibroblasts to 1% CSE, a concentration previously shown by us to activate fibroblasts (32), resulted in decreased AHR protein expression levels beginning at 2 h, the earliest time point examined (Fig. 2A). Cells cultured with control media for 8, 48, or 72 h did not exhibit a decrease in protein levels. β-actin levels were unaffected by CSE.

After ligand binding and recognition of a nuclear localization sequence, AHR is rapidly translocated to the nucleus (7). Therefore, we next examined by immunohistochemistry the ability of CSE to induce nuclear translocation in human lung fibroblasts. Unactivated lung fibroblasts showed little or no nuclear AHR (Fig. 2B, untreated). Fibroblasts treated with 1% CSE showed some weak AHR staining in the nucleus after 15 min. Exposure to 2% CSE showed moderate AHR nuclear localization at 15 min. After a 2-h exposure to CSE, there was almost exclusive and strong nuclear staining (Fig. 2B). These findings indicated that the AHR was activated by components of CSE binding to the AHR, inducing translocation to the nucleus. AHR translocation and Arnt:DNA binding was further demonstrated by EMSA analysis. Nuclear extracts obtained from fibroblasts treated with or without CSE were analyzed for DNA binding to radiolabeled oligonucleotides that correspond to the DRE. There was a time-dependent increase in DNA binding, which occurred up to 2 h posttreatment compared with cells that were untreated (Fig. 2C). This AHR:DRE band was dramatically attenuated when nuclear extracts from fibroblasts exposed to CSE were treated with 100× unlabeled probe for cold competition (Fig. 2D). Furthermore, addition of an anti-AHR antibody retarded migration of the AHR:DRE band due to increased mass associated with antibody binding (Fig. 2D). Collectively, these data clearly indicate that components found in the CSE activate the AHR signaling pathway in human lung fibroblasts.

MNF, an AHR antagonist, prevents CSE-induced nuclear translocation. The synthetic flavone MNF is a potent AHR antagonist that binds to the ligand binding site but fails to initiate the process leading to AHR nuclear uptake and Arnt dimerization (18). Therefore, we tested the ability of this compound to inhibit CSE-induced translocation of the AHR in human lung fibroblasts. As assessed by immunocytochemistry, treatment of fibroblasts with 1% CSE potently induced AHR nuclear translocation (Fig. 3C) compared with cells treated only with serum-free media (Fig. 3A). In contrast, pretreatment of the cells with 1 μM MNF before its coincubation with 1% CSE for 15 min eliminated the AHR translocation, indicating that MNF is able to act as an AHR antagonist in normal human lung fibroblasts (Fig. 3D). In control experiments, neither DMSO (Fig. 3B) nor MNF alone (data not shown) had any effect on AHR translocation.

CSE-induced increase in COX-2 and PGE2 is abrogated by antagonism of the AHR. TCDD, a potent small molecule activator of the AHR, induced expression of COX-2 in human lung fibroblasts, an effect that was abolished with MNF (Fig. 4A). Furthermore, we have recently shown that CSE induces COX-2, but not COX-1, in human lung fibroblasts (32). To further investigate whether the AHR was a pathway for CSE-induced COX-2 production, we examined the ability of known AHR antagonists to blunt the upregulation of COX-2 by CSE. Fibroblasts exposed to the AHR antagonists before CSE treatment were analyzed for COX-2 protein expression (Fig. 4B). Cells exposed to 1% CSE (Fig. 4B, top, lane 6) show an increase in COX-2 protein expression compared with cells treated with media alone (Fig. 4B, top, lane 1). In contrast, MNF, at varying concentrations (Fig. 4B, top, lanes 7–9), reduced COX-2 levels to those similar to control-treated cells. Although less effective, pretreatment of human lung fibroblasts with varying concentrations of the AHR antagonists 3’,5’-MOF (Fig. 4B, top, lanes 10–12) or 3’-DMF (lanes 13–15) resulted in a concentration-dependent decrease in COX-2. Cells exposed to DMSO or the antagonists alone did not exhibit significant amounts of COX-2 (lanes 2–5). COX-1 levels were unaffected by treatment with CSE or the AHR antagonists (Fig. 4B, bottom). Collectively, these data demonstrate that AHR antagonists diminish COX-2 protein levels.

Fibroblast strains

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Fig. 1. Human lung fibroblasts express aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (Arnt). Whole cell extracts were prepared from 4 strains of normal human lung fibroblasts (each from a different human being), and equal amounts of total protein were assayed for AHR and Arnt using Western blotting procedures as detailed in MATERIALS AND METHODS. The membrane was probed with antibodies against AHR, Arnt, or β-actin and visualized with chemiluminescence. AHR and Arnt were detected in all human lung fibroblast strains tested.
To determine whether the decrease in CSE-induced COX-2 protein levels after AHR antagonist exposure was due to changes in steady-state mRNA levels, we analyzed for changes in COX-2 mRNA by RNase protection analysis. Cells were treated with or without MNF before CSE exposure, and total RNA was isolated and subjected to $^{32}$P-labeled riboprobes against COX-2 and $\beta$-actin. COX-2 mRNA was increased in fibroblasts treated with 1 and 2% CSE (Fig. 4C, top, lanes 4 and 6) compared with incubation with media or DMSO (lanes 1 and 2). This increase in COX-2 was abrogated when the cells were treated with MNF (lanes 5 and 7), indicating that an AHR antagonist suppressed increases in steady-state COX-2 mRNA levels. Densitometry analysis of COX-2 mRNA levels reflected the changes observed in Fig. 4C, where there was a significant increase in the relative intensity of COX-2 message after treatment with 2% CSE (data not shown). These data therefore indicate that there is a decrease in steady-state COX-2 mRNA levels in cells pretreated with the AHR antagonists before CSE exposure, suggesting that the AHR pathway is integral in CSE-induced COX-2 protein and mRNA synthesis in normal human lung fibroblasts.

AHR antagonists decrease the ability of CSE to induce PGE$_2$ and mPGES-1. To evaluate whether the decrease in COX-2 mRNA and protein following AHR antagonism also yielded a concomitant decrease in PG production following smoke exposure, PGE$_2$ levels were evaluated. PGE$_2$ levels were evaluated. PGE$_2$ plays a central role in regulating inflammation and contributing to the development of carcinogenesis (41, 51). Fibroblasts were cultured as described above and serum starved before being treated with the AHR antagonists MNF, 3'-H11032, 5'-H11032-MOF, and 3'-H11032-DMF for 1 h at concentrations shown in Fig. 5. The cells were then cotreated with 1% CSE for 24 h, the cell culture supernatant was harvested, and enzyme immunoassay was performed. Cells treated with media, DMSO, and the highest concentrations of...
untreated cells (compare with resulted in an increase in nuclear staining (arrows) when compared with C. This reduction in nuclear staining (compare with 1/H9262 A).

Fibroblasts (Fig. 6A, lane 3). Furthermore, the increase in mPGES-1 following treatment with 1% CSE was abrogated by pretreatment with the AHR antagonist MNF (Fig. 6A, compare lanes 5 and 6, and Fig. 6B).

In contrast, mPGES-2 was not induced by treatment of human lung fibroblasts with IL-1β (Fig. 7, A and B). There was a slight increase in mPGES-2 levels with CSE treatment; the antagonists alone produced little PGE2. However, fibroblasts treated with 1% CSE produced >4,000 pg/ml of PGE2 in the media, a significant increase over control-treated cells. CSE had no significant effect on the synthesis of PGJ2 or PGF2α or thromboxane B2 (data not shown). All of the AHR inhibitors significantly reduced CSE-induced production of PGE2. 3',5'-MOF and 3',5'-DMF exhibited concentration-dependent inhibition of PGE2 production where the highest concentration used, 1 μM, resulted in PGE2 levels at, or below, control concentrations. To ensure that the drop in PGE2 levels was not a result of cytotoxicity, cells were treated with the highest concentration of each antagonist plus 1% CSE, and viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. For each antagonist, fibroblast viability was not affected (data not shown).

The ability of MNF to block CSE-induced increases in PGE2 led to the supposition that mPGES-1 and mPGES-2, key enzymes involved in PGE2 synthesis downstream of COX-2, may also have been affected. Little is known about human lung fibroblast expression of the mPGE synthases. To date, mPGES-2 is generally found to be constitutive, whereas mPGES-1 can be upregulated by inflammatory stimuli (36). Fibroblasts treated with 1% CSE with or without prior treatment with the AHR antagonist MNF (1 μM, 1 h) were assayed for mPGES-1 protein levels by Western blot analysis. Treatment with IL-1β or 1% CSE resulted in a 6- to 7-fold increase in mPGES-1 protein expression in normal human lung fibroblasts (Fig. 6A, lane 5, and Fig. 6B). Pretreatment of human lung fibroblasts with MNF alone reduced basal levels of mPGES-1 (Fig. 6A, lane 3). Furthermore, the increase in mPGES-1 following treatment with 1% CSE was abrogated by pretreatment with the AHR antagonist MNF (Fig. 6A, compare lanes 5 and 6, and Fig. 6B).

In contrast, mPGES-2 was not induced by treatment of human lung fibroblasts with IL-1β (Fig. 7, A and B). There was a slight increase in mPGES-2 levels with CSE treatment;
densitometric analysis revealed an approximate twofold increase above cells treated with media alone (Fig. 7B). These data together with the PGE2 results (Fig. 5) indicate that there is a concordant activation of the AHR pathway followed by upregulation of PG biosynthesis in normal human lung fibroblasts.

**DISCUSSION**

Cigarette smoking is the primary cause of lung cancer and the lung inflammatory diseases emphysema and chronic bronchitis. Smoking currently accounts for ~30% of all cancer deaths in developed countries (42). We have previously shown that the COX-2 pathway is induced by components of cigarette smoke in normal human lung fibroblasts (32), a group of cells within the lung that not only confer structural support but incite and amplify inflammation. In the present study, our data clearly show that the AHR mediates CSE-induced inflammation in human lung fibroblasts. Specifically, CSE activates the AHR pathway to induce COX-2 and PGE2; abrogation of this pathway using AHR-specific antagonists dramatically attenuates mPGES-1, COX-2, and PGE2 induction in CSE-treated normal human lung fibroblasts.

COX-2 and its PG products play an important role in the pathogenesis of lung diseases such as COPD (62), asthma (56), and lung cancer (4). Increased expression of COX-2 is seen in pulmonary biopsies from patients with COPD and in sputum and bronchial biopsies from patients with asthma (56, 62) and non-small cell lung cancers and premalignant bronchial and alveolar lesions (19, 29). Furthermore, COX-2 inhibitors substantially reduce lung cancers induced by carcinogens in mice (33, 45). COX-2 expression is rapidly induced by various growth factors, mitogens, proinflammatory cytokines, and chemokines (54). Within the lung, fibroblasts have an early and central role in inflammation. They quickly alert the body to a danger signal such as respiratory insult, infection, and tissue damage, etc. (53), and, within minutes of activation, fibroblasts begin to express immediate-early genes including COX-2 (65) and mPGES-1 (32). Importantly, fibroblasts are adjacent to, and cross talk with, epithelial cells, the cellular origin of most cancers (60, 67).

Until this and our previous study (32), little was known about the ability of CSE to induce an inflammatory response in normal human lung fibroblasts. COX activity can be induced by cigarette smoke in cell types such as rat alveolar macrophages (20), neuronal cells (59), human histiocytic lymphoma cells, oral epithelial cells (23), and human lung epithelial cell lines (50), with many of these being induced through the NF-κB pathway (2, 50, 59). Although most studies indicated that NF-κB is a key regulator for COX-2 upregulation in response to proinflammatory stimuli, we found that blocking the NF-κB pathway did not result in complete COX-2 inhibition in human lung fibroblasts (32). This suggested there was more than one pathway impacting COX-2 expression in human lung fibroblasts exposed to CSE. We therefore sought to examine the role of the AHR in cigarette smoke-induced inflammation in human lung fibroblasts.

![Fig. 5. AHR antagonists inhibit CSE-induced prostaglandin E2 (PGE2) production. Normal human lung fibroblasts were serum starved for 48 h before being pretreated with the AHR antagonists MNF, 3',5'-MOF, and 3'-DMF for 1 h, followed by treatment with 1% CSE for 24 h. Supernatants were harvested and analyzed for PGE2 levels. PGE2 levels were significantly induced with 1% CSE, an effect blunted by all three AHR antagonists. Results are presented as picograms/milliliter ± SD. Experiments were done in triplicate and repeated at least 3 times. **P < 0.01 vs. negative control. *P < 0.05 indicates statistical significance between fibroblasts treated with CSE relative to fibroblasts treated with CSE and AHR antagonists.](image1)

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CSE is a complex mixture of >5,000 chemicals, including nitrosamines and polycyclic aromatic hydrocarbons such as benzo[a]pyrene (9). The AHR can be activated by cigarette smoke in Hepa1c1c7 cells (9, 12) as well as in vivo (8). The AHR agonist TCDD has been shown to rapidly induce the COX-2 pathway in cell types such as the Madin-Darby canine kidney cells, with significant increases in both COX-2 protein and mRNA (28), and we have determined that TCDD induces COX-2 in human lung fibroblasts (Fig. 4A). Furthermore, a constitutively active AHR is able to induce stomach tumors in mice (1). To establish whether the AHR represents a dominant pathway in the human lung system, we were first interested in determining the presence of AHR and Arnt protein in human lung fibroblasts. We observed that the AHR and Arnt were present in all human lung fibroblast strains tested (Fig. 1). Because the presence of the protein does not necessarily indicate activation by CSE, we tested the ability of CSE to activate the AHR in vitro. CSE induced downregulation of the AHR as early as 2 h post-CSE exposure, and this decrease persisted for the longest time point examined, 72 h (Fig. 2A). Similar to our findings, exposure of mouse hepatoma cells to TCDD in vitro results in a loss of AHR protein for at least 72 h after exposure (13). It is now widely established that the AHR is proteolytically degraded via ubiquitination rapidly after ligand-dependent activation (46), indicating that activation of the AHR by components of CSE induces degradation of the receptor.

To further substantiate activation of the AHR by cigarette smoke in human lung fibroblasts, we tested for the ability of CSE to induce nuclear translocation of the AHR. In its unactivated state, the AHR is a cytosolic protein. Upon ligand binding, the AHR rapidly translocates to the nucleus. We observed significant migration of the AHR protein to the nuclei of normal human lung fibroblasts (Fig. 2B) and were also able to show AHR:Arnt:DRE binding using an EMSA (Fig. 2C). Finally, we were able to show functionality of the AHR system by blocking COX-2 protein and mRNA upregulation with AHR-specific small molecule inhibitors (Fig. 4). These data were bolstered by a decrease in both mPGES and PGE_2 synthesis in cells treated with AHR inhibitors (Figs. 5 and 6). Together, our data indicate that CSE activates human lung fibroblasts, inducing the expression of mPGES, COX-2, and PGE_2, and that the dominant pathway for this induction involves the AHR.

Our data are in contrast to a study done by Gradin et al. (17). Here, researchers found that fibroblasts obtained from neonatal foreskin did not respond to TCDD, as indicated by a lack of inducibility of cytochrome P-4501A1 mRNA expression. However, our report is the first to examine the AHR response to CSE in adult human primary lung fibroblasts using markers of inflammation as outcome measures. It is therefore plausible that the AHR response to dioxin vs. other environmental toxicants may vary between fibroblasts isolated from different anatomical regions or may be age related. Indeed, we have shown that fibroblasts from the orbit of the eye and female reproductive tract exhibit considerable heterogeneity in their proinflammatory response (25–27, 49). A subsequent study indicated that the dermal fibroblasts contained a putative repressor protein termed the AHR repressor (AHRR), which heterodimerizes with Arnt, binds to the DRE domain in the promoter of the pertinent gene, and blocks TCDD-mediated AHR gene activation (16). This repressor, when overexpressed, may inhibit AHR/Arnt heterodimerization (15) and account for the low inducibility of the Cyp family (58) previously shown. We have identified AHRR mRNA in three strains of human lung fibroblasts (unpublished observations). The role of the AHRR in regulating the immunological response in human lung fibroblasts has not yet been determined.

This is the first report to show regulation of mPGES-1 levels by the AHR (Fig. 6A). There are two forms of PGE synthase, mPGES-1 and mPGES-2. mPGES-2 is constitutively expressed and is not typically induced by inflammation (36). In accordance with this, IL-1β was not able to increase the expression of this synthase in human lung fibroblasts (Fig. 7B) or A549 epithelial cells (data not shown). In contrast, the expression of mPGES-1, which can be upregulated by proinflammatory stimuli such as IL-1β, is increased in malignancies of the gastrointestinal (22, 40) and male reproductive (14) tracts as well as lung cancer (64). Both CSE and IL-1β upregulated mPGES-1 levels in human lung fibroblasts (Fig. 6). Pretreatment with the AHR antagonist MNF reduced both basal and CSE-induced mPGES-1 levels. Although an endogenous AHR ligand remains to be elucidated, the reduction in basal mPGES-1 expression by AHR antagonism may reflect inhibition of endogenous AHR activity in human lung fibroblasts. Collectively, these results indicate that increased mPGES-1 expression following treatment with CSE may further contribute to the proinflammatory milieu of the lung in COPD and emphysema and contribute to the progression of lung cancer.
In conclusion, the results presented here clearly indicate that the AHR is a dominant pathway that mediates mPGES-1, COX-2, and PGE2 production in human lung fibroblasts. Targeting the AHR may represent a viable treatment option for chronic inflammatory conditions in the lung that plague many of the 80 million current and former smokers today in the United States. The potential of selective chemoprevention by targeting the AHR pathway to downregulate COX-2 and its PG products is an exciting possibility. Targeting the AHR pathway could lead to a reduction in smoking-induced inflammatory diseases such as COPD as well as lung cancer.

REFERENCES


