Arylhydrocarbon Receptor Activation in NCI-H441 Cells and C57BL/6 Mice

Possible Mechanisms for Lung Dysfunction

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The arylhydrocarbon receptor (AhR) is known for its ability to bind aromatic-containing compounds, which starts a molecular cascade involving the induction of cytochrome P450s and inflammatory cytokines. Our hypothesis is that many inhaled environmental toxicant components activate these inflammatory pathways via an initial binding to the AhR. To test this possibility, we treated Clara cell-derived NCI-H441 cells with the AhR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and demonstrated that AhR activation increased the expression of both cytochrome P450s and inflammatory markers. We also found increased mucin 5AC production with TCDD treatment. Similar results were observed in NCI-H441 cells treated with urban dust particles. Mucin 5AC expression was highly correlated with increased-expression cyclooxygenase-2 and IL-1 β , thus implicating these two inflammatory markers as possible conduits for AhR-mediated mucin production. We hypothesize that this increase in mucin 5AC production is a result of inflammation-induced differentiation of our epithelial cell to a mucin-producing cell. This theory is supported by morphological changes observed in the cells, as well as decreased expression of Clara cell secretory protein (CC10). In an *in vivo* C57BL/6 mouse model, TCDD increased expression of inflammatory cytokines, mucin 5AC, and a number of matrix metalloproteases in whole-lung samples. These changes were not seen in mice in which AhR signaling was repressed. These markers from the whole-lung samples have been correlated to onset of bronchitis, asthma, small airways disease, and fibrosis, and their increased expression further implicates AhR activation in producing the molecular environment for the development of lung injury to occur.

Keywords: arylhydrocarbon receptor; 2,3,7,8-tetrachlorodibenzo-p-dioxin; mucin; air pollution

Many inhaled toxicants, such as benzo(a)pyrene, from cigarette smoke and nitrated PAHs from particulate matter have been shown to activate this arylhydrocarbon receptor (AhR) system in the lung (1–4). This is not surprising, because the lung contains the highest concentration of AhRs compared with other organs (5–7). To study the AhR system, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) remains a popular choice as a probe to investigate the molecular aspects of this system. The choice of TCDD is understandable, as it is regarded as the most toxic congener of the dioxin-type chemicals due to its metabolic stability and acknowledged toxicities (8). For this reason, TCDD was used as a model compound for AhR activation in our experiments.

It has been shown that, through these pathways, transforming growth factor (TGF)- α (9, 10) is the major autocrine factor activated. Although the exact mechanism has yet to be determined, increased TGF- α signaling, in itself, has been implicated in a wide variety of lung dysfunctions, including pulmonary fibrosis (11, 12), up-regulation of mucin genes through inflammation (13, 14), and prognosticators of lung tumors (15–17). TGF- α exerts its effect by activating the epidermal growth factor receptor (EGFR), src kinase. This activation starts a cascade of possible pathways, such as the I κ B pathway, which regulates NF- κ B, protein kinase (PK) A, which regulates the cyclooxygenase (COX)-2/prostaglandin cascade, and members of the CCAAT/enhancer-binding protein (C/EBP) family.

Previously, our laboratory has shown that increased C/EBP- β transcription after dioxin exposure is regulated by a cAMP/PKA-mediated process (18). The implication of this pathway elucidates other possible avenues for dioxin-mediated lung dysfunction—in particular, the possibility that IL-1 β , a strong COX-2 activator, as well as marker of TCDD oxidative stress (19, 20), acts as a conduit between AhR and COX-2 activation. This mechanism is supported by a recent study that proposes that acute lung injury via IL-1 β results from the activation of prostenoids, such as prostaglandin E2 (21), which activate mucin secretion.

Despite the copious evidence that these molecular factors are involved in AhR-mediated lung dysfunction, the exact molecular mechanism for up-and down-regulation of these transcription factors has not yet been determined. Many important lung proteins are regulated by these transcription factors, and AhR activation can induce changes that can effect their expression as well. For example, matrix metalloproteases (MMPs) have been shown to be up-regulated by both C/EBP- β and TNF- α (22). Increased production of these proteins leads to decreased extracellular matrix formation, and subsequently impairs the lungs ability for proper morphogenesis, organogenesis, and tissue repair. This, in turn, may lead to pulmonary fibrosis and lung remodeling, which have been attributed to both inhaled toxicants and increased TGF- α expression (11, 23). In another example, many proinflammatory cytokines have been shown to be up-regulated by C/EBP- β and TNF- α . These proinflammatory cytokines have been shown to increase mucin production and hypersecretion, a major lung complication that can lead to asthmatic conditions, fibrotic conditions, and small airways disease. This pathway is supported by studies that have linked EGFR activation to increased mucin production in epithelial cells (14). Secretory proteins, such as surfactant protein (SP)-A and Clara cell secretory protein (CCSP) appear to be highly regulated by AhR-affected transcription factors. AhR-mediated up-regulation of TNF- α and down-regulation of C/EBP- α both lead to decreased formation of SP-A (6, 22). SP-A is the major lung surfactant-associated protein that mediates local defense against pathogens and modulated inflammation in the alveolus. The down-regulation of this protein may provide a possible explanation for increased immune disease often seen with environmental toxicants, as well as TCDD exposure (24,

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25). CCSP, also known as CC10, is a cell-specific differentiation marker for the bronchiolar Clara cells. It has been shown to be up-regulated by EGFR stimulation (26), possibly via C/EBP- α coactivation (27, 28), and may be another marker for AhR-mediated changes in lung morphology.

Although it is obvious that there are many interesting, complex mechanisms involved in the activation of the AhR in the lung, the focus of this study was not the exact mechanism of AhR activation, but rather the consequences of AhR activation on the expression of key inflammatory cytokines and lung-specific proteins. This article illustrates that AhR activation promotes molecular mechanisms that can lead to lung remodeling, inflammation, and hypermucus secretion in both cellular and animal models. The establishment of these mechanisms can lead to future studies on lung disease pathways by various AhR-activating environmental insults, as well as the development of possible anti-AhR-based therapeutics.

MATERIALS AND METHODS

Cell Culture

NCI-H441 cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 containing 10% FBS (Gemini, Woodland, CA).

Animals and Treatment

Male C57BL/6J (C57BL/6) mice (8 wk old) were purchased from Jackson Laboratory (West Sacramento, CA). NLS-strain (AhR^{NLS}) mice with a mutation in the AhR nuclear localization domain were generated and kindly provided by Christopher Bradfield and coworkers from the McArdle Laboratory for Cancer Research at the University of Wisconsin (29). Mice were housed (3–4 per cage) in a selective, pathogen-free facility, and a humidity- and temperature-controlled room. The animals were maintained on a 12 hour/12 hour light/dark cycle, and had free access to water and food, according to the guidelines set by the University of California at Davis.

Cellular TCDD Exposure

The 3-, 8-, 24-hour experiments. Cells were plated (50,000) into 60-mm dishes. After 24 hours, the media were refreshed, and TCDD (10 nM final concentration) was added to cells either immediately or after 16, 18, or 21 hours (for 24-, 8-, 6-, and 3-h exposure times, respectively). All cells were then harvested 24 hours after the previous media change, and mRNA was immediately extracted.

The 1-, 3-, 5-day experiments. NCI-H441 cells were plated and 24 hours later, media were changed and fresh media containing TCDD was then added. The 1-day plate was extracted for mRNA or stained 24 hours later. This process was also repeated for the 3- and 5-day plates, such that the last media change with or without TCDD occurred 24 hours before each harvesting.

Cellular Urban Dust Particle Exposure

National Institute of Standards and Technology Standard Reference Material (SRM) 1649, an atmospheric particulate material collected in an urban area, was purchased from National Institute of Standards and Technology (Gaithersburg, MD). Stock solutions of particles were prepared by suspension in autoclaved distilled water, and by ultrasonication for 2 minutes at maximum power (100 W). Particles were used at 10 $\mu\text{g}/\text{cm}^2$, equivalent to 50 $\mu\text{g}/\text{ml}$. Urban dust particle (UDP) was added in a similar manner as TCDD for 1-, 3-, and 5-day exposure.

Animal TCDD Exposure

Mice were intraperitoneally injected with a single dose 15 $\mu\text{g}/\text{kg}$ (C57BL/6) or 100 $\mu\text{g}/\text{kg}$ (NLS) of TCDD dissolved in a mixture of corn oil and acetone. Food and water was given *ad libitum*. C57BL/6 Mice were killed after a 1-, 7-, or 30-day exposure period. NLS mice were killed after a 7-day exposure period.

Cellular mRNA Extraction

mRNA was extracted from the cells using the RNeasy kit (QIAGEN, Valencia, CA).

Mouse Lung mRNA Extraction

At the end of the 1-, 7-, or 30-day treatment period, TCDD-treated and control mice were killed using cervical dislocation, and the lungs were stored in RNAlater solution at -80°C .

mRNA was extracted from the lungs by homogenizing the whole lung in 2 ml of Trizol. Then, 1 ml of the homogenate solution was extracted by 200 ml of chloroform. The resulting aqueous layer was isolated and mixed with 70% ethanol. mRNA was then isolated from this ethanol solution using the RNeasy kit.

cDNA Preparation

The isolated mRNA was transformed into cDNA using a QIAGEN Omniscript reverse-transcriptase kit.

Real-Time RT-PCR

Using primers designed for transcription factors, secretory proteins, structural and proteolytic proteins, and markers of inflammation, RT-PCR was performed on the cDNA using a QIAGEN QuantiTech SYBR-green RT-PCR kit and the Roche Lightcycler PCR system (Roche, Indianapolis, IN). mRNA expression was normalized by glyceraldehyde 3-phosphate dehydrogenase expression for mouse samples, and β -actin for cultured human cell samples.

Primer Design

Primer design was accomplished by literature searches for DNA sequences for target proteins and the Primer3 primer design program obtained from the Broad Institute Website (<http://www.broad.mit.edu/>).

Mucin Staining

The cells were fixed in 10% formaldehyde solution and stained for mucin using a Wigert's iron Hematoxylin-based rapid mucin staining kit (Polyscience Inc., Warrington, PA).

Figure 1. Induction of cytochrome P450-1A1 (CYP1A1), COX-2, and monocyte chemoattractant protein (MCP)-1 mRNA measured by quantitative RT-PCR. NCI-H441 cells were exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; 10 nM) for 3 hours to 5 days before mRNA extraction. * $P < 0.05$ versus control vehicle. Results are presented as means ($\pm$ SD).

Figure 2. Induction of mucin 5AC and Clara cell secretory protein (CCSP) mRNA measured by quantitative RT-PCR. NCI-H441 cells were exposed to TCDD (10 nM) for 3 hours to 5 days before mRNA extraction. * $P < 0.05$ versus control vehicle. Results are presented as means ($\pm$ SD).

Data and Statistical Analysis

All RT-PCR experiments were repeated a minimum of three times, and results were expressed as means ($\pm$ SD) with respects to mRNA expression of gene of interest versus β -actin or glyceraldehyde 3-phosphate dehydrogenase. Statistical differences were determined by Student's *t* test, and the Bonferroni *post hoc* test was used for the analysis of significance between pairs of mean values. Correlation analysis was by calculation of Pearson's *r* value.

RESULTS

Effect of AhR Activation on NCI-H441 mRNA Expression

We started our investigation by exposing NCI-H441 cells to TCDD for a variety of time points from 3 hours to 5 days (Figure 1). Cytochrome P450-1A1 (CYP1A1) was the first gene analyzed, as it is the pre-eminent marker for AhR activation (30). As expected, TCDD strongly induces CYP1A1 expression. In addition, the increased expression of the inflammatory cytokine, COX-2, and monocyte chemoattractant protein (MCP)-1 was also observed, and these results support the hypothesis that AhR activation leads to increased expression of inflammatory markers. Small increases (normally less than twice control) were also seen for TGF- α , IL-1 β , TNF- α , and EGFR (data not shown). To investigate possible links between these inflammatory markers and lung-specific proteins, SP-A

and mucin (1, 2, (5AC, 5B, and 6) expression was also investigated. Of this group, only mucin 5AC demonstrated measurable increases by TCDD treatment (Figure 2). The overexpression of mucin 5AC by NCI-H441 cells has important implications, given the location and function of Clara cells in the pulmonary epithelium. Increased mucin production, especially in small airways, may lead to increased onset of chronic diseases, such as chronic obstructive pulmonary disease. Although the exact mechanism of mucin production from Clara cells has yet to be clearly defined, it has been hypothesized that Clara cells can differentiate into high mucin-containing goblet cells when exposed to TNF- α , a cytokine shown to be up-regulated by AhR activation (14). This possibility is supported by Figure 2, which illustrates that TCDD exposure does lead to a decrease in the Clara cell marker CCSP mRNA expression over the same time period.

To confirm the role of AhR in these observations, similar experiments were performed in the presence of the AhR inhibitor, 3' methoxy-4'-nitroflavone for the 1-, 3-, and 5-day exposure studies (Figure 3). The addition of this specific AhR inhibitor reduced TCDD-mediated mRNA expression of CYP1A1 and mucin 5AC to control levels. In addition, TCDD-mediated COX-2 mRNA expression was reduced by roughly 50%.

To confirm our gene expression results, we repeated our NCI-H441-TCDD exposure experiment, fixed the cells with formaldehyde, and stained for mucin proteins (Figure 4). Both Figures 2 and 4 demonstrate that TCDD treatment does lead to either increased mucin production in NCI-H441 cells, or the differentiation of this cell to another mucin-producing cell. This second possibility is supported by morphological changes in TCDD-treated cells seen in Figure 4. To gain possible insight to molecular mechanisms involved in the increased expression of mucin 5AC by AhR activation, correlation analysis of mRNA expression across all five time points was conducted for possible inflammatory and lung-specific markers (Table 1). This analysis compared TCDD-mediated increases over control for various markers compared with increases seen by mucin 5AC over the same time frame. Using this method, a number of markers (especially CYP1A1, IL-1 β , and COX-2) demonstrated a fairly consistent correlation with mucin 5AC. In addition, CCSP showed a strong negative correlation with mucin 5AC expression.

Effect of UDP on AhR-Mediated Expression

In previous experiments with other cell lines in our laboratory (4), common air pollutants were able to stimulate inflammatory responses through possible AhR activation. To investigate this possibility in our cellular system, NCI-H441 cells were treated with atmospheric particulate matter collected from an urban core area. These UDP are often coated with aromatic hy-

Figure 3. Effect of 3' methoxy-4'-nitroflavone (MNF) on TCDD-mediated mRNA expression of CYP1A1, COX-2, and MUC5AC measured by quantitative RT-PCR. NCI-H441 cells were exposed to TCDD (10 nM) and MNF for 3 hours to 5 days before mRNA extraction. * $P < 0.05$ versus control vehicle; ^a $P < 0.05$ MNF versus no MNF. Results are presented as means ($\pm$ SD).

Figure 4. Mucin staining of NCI-H441 treated with TCDD. NCI-H441 cells were treated with TCDD (10 nM) or the dioxane vehicle for between 1 and 5 days. Cells were fixed in 10% formaldehyde after Days 1, 3, or 5, and stained for mucin using a Wiggert's iron hemotoxylin-based rapid mucin staining kit (Polyscience Inc.).

drocarbons, which activate the AhR (31). Figure 5 illustrates that UDP, similar to TCDD, up-regulates CYP1A1, COX-2, and mucin 5AC. Furthermore, the effects of 3' methoxy-4'nitroflavone on these markers on UDP were remarkably similar with complete inhibition of mRNA overexpression of CYP1A1 and mucin 5AC, and an approximate 50% reduction of COX-2 expression. These particular results demonstrate that the AhR may act as an important transducer of air pollution-related inflammation and lung disease.

***In Vivo* C57BL/6 Mouse Studies with TCDD**

For these experiments, C57BL/6 mice were injected with TCDD (15 µg/kg) intraperitoneally or the corn oil vehicle. After 1, 7, or 30 days, the animals were killed, and mRNA was extracted from the whole lung and measured by RT-PCR. Increased CYP1A1 expression (Figure 6) is indicative of AhR activation in this animal model system over the treatment period. The up-regulation of the inflammatory cytokines, COX-2, IL-1β, and MCP-1 (Figure 6), is indicative of a systemic increase of inflammation, induced possibly by increased macrophage and neutrophil infiltration.

Another inflammatory cytokine up-regulated by TCDD in our C57BL/6 animal model is TNF-α (Figure 7). Previous studies have indicated that TNF-α expression has a negative effect on SP-A, and this is indeed observed in our study (Figure 7). The decrease of this protein leaves the lung more susceptible to immune disease, and may also increase the injury caused by other up-regulated inflammatory cytokines. Also described previously is the role of TNF-α in the differentiation of Clara cells to mucin-producing goblet cells. The mRNA expression of mucin 5AC and CCSP in our treated C57BL/6 mice demonstrates a trend of decreased CCSP (albeit not statistically significant) with increased mucin 5AC production supports the possibility of this differentiation. These results are comparable with our NCI-H441 cell culture studies.

Another consequence of increased TNF-α expression is associated with the ability of this cytokine to increase the production of MMPs, which break down the extracellular matrix that supports the basic structure of lung morphology. Figure 8 illustrates the ability of TCDD to up-regulate the production of MMPs in our C57BL/6 animal model system.

***In Vivo* NLS Mouse Studies with TCDD**

To establish that *in vivo* TCDD effects in the C57BL/6 mouse are AhR mediated, we used the AhR^{NLS} (NLS) strain, developed by the Bradford Laboratory (29). This strain expresses a functioning AhR, which contains a mutation in the nuclear locali-

zation domain. The result of this mutation is the expression of an AhR, which binds its common agonists, yet does not translocate and activate the dioxin response element (DRE) in the nucleus. As expected, NLS mice were not responsive to TCDD, as demonstrated by the lack of an effect on CYP1A1 expression, a key AhR/DRE marker (Figure 9). In addition, contrary to the wild-type C57BL/6, TCDD was unable to increase expression of the inflammatory cytokines, COX-2 and IL-1β (Figure 9), in the NLS strain, thus implicating a possible role for AhR/DRE in their expression. Interestingly, TCDD up-regulates MCP-1 in both of these strains (Figure 9), indicating a non-AhR/DRE-mediated pathway for its expression. As for the lung secretory proteins (Figure 10), mucin 5AC overexpression in the wild-type mice was eliminated in the NLS mice. In addition, the NLS mice demonstrated decreased TCDD-mediated CCSP suppression when compared with the wild-type C57BL/6. These data support our hypothesis that AhR/DRE activation may be involved in increased proportion of mucin-producing cells. The translocation of AhR also appears to be involved in the overexpression of the MMPs (Figure 11). In this study, TCDD had a reduced (MMP-12) or completely opposite (MMP-13) effect in the NLS compared with wild-type, thus indicating a possible role of the AhR/DRE activation for effecting lung morphogenesis and tissue repair. In addition to the response to TCDD challenge, this supplemental study revealed that, although there was little difference with most genes, the NLS model had a lower basal expression of COX-2, mucin 5AC, and CCSP when compared with β-actin expression.

TABLE 1. PEARSON'S CORRELATION DATA WITH MUCIN 5AC mRNA EXPRESSION

Parameter	Pearson's <i>r</i> Value
COX-2	0.969
CYP1A1	0.936
IL-1β	0.870
EGFR	0.815
TGF-β	0.772
NF-κB	0.422
TNF-α	0.067
MCP-1	0.304
CCSP	0.936

Definition of abbreviations: CCSP, Clara cell secretory protein; COX, cyclooxygenase; CYP1A1, cytochrome P450-1A1; EGFR, epidermal growth factor receptor; MCP, monocyte chemoattractant protein; TGF, transforming growth factor.

Figure 5. Effect of MNF on TCDD-mediated mRNA expression of CYP1A1, COX-2, and MUC5AC measured by quantitative RT-PCR. NCI-H441 cells were exposed to TCDD (10 nM) and MNF for 3 hours to 5 days before mRNA extraction. * $P < 0.05$ versus control vehicle; ^a $P < 0.05$ MNF versus no MNF. Results are presented as means ($\pm$ SD).

DISCUSSION

The human adenocarcinoma NCI-H441 cell line is thought to be derived from a bronchiolar Clara cell. Clara cells are a non-ciliated epithelial cell line that predominates the surface area in small (<2 mm) airways. They are secretory in nature, and produce a wide variety of glycoproteins, such as mucins and SP-A. Along with their secretory properties, they have been implicated in many diverse roles, ranging from a progenitor cell to a detoxifier. Of particular interest to our study, Clara cells have been identified as being the most sensitive in responding to AhR stimuli (32). In our cellular studies described above, after CYP1A1, mucin 5AC was our most consistently expressed gene after AhR activation in the NCI-H441 cell line. The observation of increased mucin 5AC mRNA expression and mucin staining in the NCI-H441 cell line after TCDD exposure, we believe is a clear indicator of a molecular pathway for lung dysfunction. Similar results obtained with UDP further implicate these pathways as possible mechanisms for deleterious effects of airborne pollutants; especially particles that contain polyaromatic hydrocarbons. Hypersecretion is associated with a number of inflammatory lung diseases, including asthma, cystic fibrosis, bronchitis, and chronic obstructive pulmonary disease. Under these conditions, it is believed that

increased mucin production leads to increased obstruction of small airways, a condition that may be responsible for increased difficulty in breathing in these disease states. Excess mucin production has been molecularly linked to the conditions of goblet cell hyperplasia in humans or goblet cell metaplasia in mice. This correlation, along with the general opinion that most mucin production occurs in these goblet cells, leads us to hypothesize that AhR activation doesn't directly increase mucin mRNA production in Clara cells, but rather increases the potential for Clara cells to differentiate into mucin-producing goblet cells. It must be noted that a number of studies do indicate that Clara cells can produce mucin 5AC without differentiation (33, 34). The data that indicated lower CCSP expression (Figure 2), as well as morphological changes (Figure 4), however, do support a possible loss of Clara Cell phenotype. Based on previous studies (14, 35), and described by Nadel and colleagues (36), Clara cells can be differentiated to mucin production by TNF- α , which increases EGFR expression and TGF- α , which activates a differentiation cascade. Correlation analysis of mucin 5AC expression (Table 1) versus these markers validates that this mechanism is reasonable. In addition, there appears to be a strong association of mucin 5AC induction by AhR via an IL-1 β -to-COX-2-mediated pathway.

Figure 6. Effect of TCDD on expression of inflammatory markers in C57BL/6 mouse lungs measured by quantitative RT-PCR. Mice were injected with TCDD (15 μ g/kg, intraperitoneal). After 1, 7, and 30 days, animals were killed, and mRNA was extracted from the isolated lungs * $P < 0.05$ TCDD versus control. Results are presented as means ($\pm$ SD).

Figure 7. Effect of TCDD on expression of TNF- α and lung-specific markers in C57BL/6 mouse lungs measured by quantitative RT-PCR. Mice were injected with TCDD (15 μ g/kg, intraperitoneal). After 1, 7, and 30 days, animals were killed and mRNA was extracted from the isolated lungs. * $P < 0.05$ TCDD versus control. Results are presented as means ($\pm$ SD).

Figure 8. Effect of TCDD on matrix metalloproteases expression in C57BL/6 mouse lungs measured by quantitative RT-PCR. Mice were injected with TCDD (15 μ g/kg, intraperitoneal). After 1, 7, and 30 days, animals were killed, and mRNA was extracted from the isolated lungs. * $P < 0.05$ TCDD versus control. Results are presented as means ($\pm$ SD).

Although cell culture models are useful for measuring the direct effects of AhR activation in gene expressions, cellular-cellular interactions can greatly influence the effects of these compounds. Therefore, we initiated *in vivo* experiments in the C57BL/6 mouse strain to study the effects of TCDD exposure on whole-lung gene expression, and provide us with a “snapshot” of the average effect of AhR activation on the global lung population. Similar to the cellular data, TCDD treatment increased CYP1A1 expression in the lung (Figure 6). This is not surprising, given the universality of this receptor. This figure also illustrates slight yet significant increases in COX-2 and IL-1 β expression. Although not as dramatic as results seen in our *in vitro* studies, the chronic higher expression of these markers indicates a constant state of increased inflammation, with the possibility of the repair/defense mechanism counteracting their expression. MCP-1 and TNF- α (Figure 7), on the other hand, demonstrated a more consistent effect of TCDD exposure than those seen in the NCI-H441 experiments, illustrating the possibility that AhR activation may be occurring in other cell types. This is not a surprising result, given the numerous cellular-cellular interactions required in this heterogeneous organ. Figure 7 also illustrates the effect of TCDD treatment on lung-specific proteins in whole-lung mRNA expression. Similar to results seen in the cell culture studies, there was, in general, a decrease in the expression of CCSP with increases in mucin 5AC production, which again illustrates a possible differentiation of Clara cells to a more mucin-producing cell line. However, similar to COX-2, the effects were not as dramatic as

in the cell culture studies, due to possible repair/defense mechanism or dilution of the results by cells that normally do not express mucin. The increase of MMP expression in the whole lung after TCDD treatment is shown in Figure 8. These proteins are importance in the regulation of the extracellular matrix, which supports the basic lung structure. Although the increased expression of these MMPs does not exactly demonstrate lung dysfunction, these conditions may impair the lungs ability for proper morphogenesis, organogenesis, and tissue repair, and may be an antecedent for future lung remodeling. As expected, NLS mice were not responsive to TCDD, as demonstrated by the lack of an effect on CYP1A1 expression, and up-regulation of key inflammatory cytokines, COX-2 and IL-1 β (Figure 9), in the NLS strain. These results implicate a possible role for AhR/DRE in their expression. This trend continues for mucin 5AC (Figure 7) and MMP-13 (Figure 11), demonstrating a possible role of AhR activation for increased mucus and lung remodeling proteins. For a couple of markers, CCSP (Figure 10) and MMP-12 (Figure 11), TCDD effects are diminished, but not eliminated, indicating the possible differences of homeostatic mechanisms. For example, the NLS strain control itself has a much lower mRNA expression of CCSP than the wild-type. Interestingly, the similarity of the effect of TCDD on MCP-1 expression (Figure 9) in both strains supports the possibility of this gene being regulated by AhR activation via a non-DRE mechanism (37).

Conclusions

These results support a basic premise that, in both cell culture and animal models, AhR activation (via TCDD) modifies gene expression for inflammatory cytokines, MMPs, and mucin production. These results are consistent with changes associated with a variety of lung diseases, such as bronchitis, asthma, small airway disease, and lung remodeling (fibrosis). In our *in vitro* Clara cell experiments, it appears that AhR activation in this cell type activates an IL-1 β -to-COX-2-mediated process,

Figure 9. Comparison of inflammatory marker expression between C57BL/6 and arylhydrocarbon receptor, NLS strain (AhR^{NLS}), mouse lungs measured by quantitative RT-PCR. C57BL/6 mice and AhR^{NLS} mice were treated intraperitoneally with 15 μ g/kg and 100 μ g/kg TCDD, respectively. After 7 days, animals were killed, and mRNA was extracted from the isolated lungs. * $P < 0.05$ TCDD versus control. Results are presented as means ($\pm$ SD).

Figure 10. Comparison of expression of lung-specific proteins between C57BL/6 and AhR^{NLS} mouse lungs measured by quantitative RT-PCR. C57BL/6 mice and AhR^{NLS} mice were treated intraperitoneally with 15 μ g/kg and 100 μ g/kg TCDD, respectively. After 7 days, animals were killed, and mRNA was extracted from the isolated lungs. * $P < 0.05$ TCDD versus control. Results are presented as means ($\pm$ SD).

Figure 11. Comparison of matrix metalloproteinase expression between C57BL/6 and AhR^{nl} mouse lungs measured by quantitative RT-PCR. C57BL/6 mice and AhR^{nl} mice were treated intraperitoneally with 15 μ g/kg and 100 μ g/kg TCDD, respectively. After 7 days, animals were killed, and mRNA was extracted from the isolated lungs. * $P < 0.05$ TCDD versus control. Results are presented as means ($\pm$ SD).

which leads to increased mucin production. This process might be facilitated via differentiation of the Clara cell to a mucin-producing, goblet-like cell phenotype.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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