GENETIC ABLATION OF THE ARYL HYDROCARBON RECEPTOR POTENTIATES CIGARETTE SMOKE-INDUCED OXIDATIVE STRESS AND APOPTOSIS

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This work is dedicated to my mother, who died of breast cancer in 2005. Given the putative roles for both the antioxidant response as well as the AhR in cancer, it is my hope that some of the research contained herein will be of use to improve medical outcomes for those who are suffering.

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Abstract

The aryl hydrocarbon receptor (AhR) is well described as the mediator of toxicological responses to environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Following binding of ligand, the AhR translocates from the cytoplasm to the nucleus and binds to the dioxin response-element (DRE), thereby activating a battery of genes coding for phase I and II detoxification pathways. Recent efforts have been focused on elucidating the endogenous roles for this ubiquitously expressed receptor/transcription factor. It has been shown that the AhR has anti-inflammatory and anti-apoptotic roles against cigarette smoke, a source of free radicals and reactive oxygen species that cause apoptosis in lung structural cells. Hence, we hypothesized that the AhR may have a protective role against apoptosis by regulating oxidative stress. Using primary lung fibroblasts derived from $AhR^{+/+}$ and $AhR^{-/-}$ mice as well as A549 human lung adenocarcinoma cells deficient in AhR expression (A549-AhR^{ko}), we show that $AhR^{-/-}$ fibroblasts and A549-AhR^{ko} cells have a significant increase in cigarette smoke extract (CSE)-induced oxidative stress compared to wild-type. Similarly, we find that there is decreased cell viability in AhR-deficient cells following CSE treatment, compared to wild-type. The mRNA expression of key antioxidant genes *Nqo1* and *Srxn1* were also strongly upregulated in $AhR^{+/+}$ cells, with significantly less induction in $AhR^{-/-}$ fibroblasts. In order to determine the manner in which Srxn1 and Nqo1 are regulated by the AhR, lung fibroblasts derived from mice that express an AhR incapable of binding to the DRE ($AhR^{DBD/DBD}$) were used. *Nqo1* levels were significantly lowered following CSE exposure to $AhR^{DBD/DBD}$ fibroblasts compared to wild-type, while the expression of Srxn1 is upregulated by CSE in both $AhR^{DBD/DBD}$ and wild-type cells. Thus, our findings indicate that *Nqo1* expression is governed by the AhR in a DRE-binding dependent manner, whereas *Srxn1* may be non-genomically

regulated. These findings may underscore a protective antioxidant mechanism for the AhR in defense against CSE-induced oxidative stress.

Résumé

Le récepteur des hydrocarbures aromatiques (AhR) est connu comme étant le médiateur des effets toxicologiques des contaminants environnementaux tels que la 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD). Une fois liée au ligand, l'AhR se déplace du cytoplasme vers le noyau et se lie à l'élément-réponse de la dioxine (DRE), activant ainsi une batterie de gènes codant pour les voies de détoxification de phase I et II. Des efforts ont été récemment faits pour élucider les rôles endogènes de ce facteur de récepteur/transcription ubiquitaire. Il a été démontré que l'AhR joue un rôle anti-inflammatoire et anti-apoptotique après contact avec la fumée de cigarette, source de radicaux libres et de dérivés réactifs de l'oxygène provoquant l'apoptose des cellules structurales pulmonaires. Par conséquent, nous avons émis l'hypothèse que l'AhR pourrait avoir un rôle protecteur contre l'apoptose en régulant le stress oxydatif. En utilisant des fibroblastes pulmonaires primaires dérivés de souris $AhR^{+/+}$ et $AhR^{-/-}$ ainsi que des cellules d'adénocarcinome pulmonaire humain A549 déficientes en AhR (A549-AhR^{ko}), nous avons montré que les fibroblastes $AhR^{-/-}$ et les cellules A549-AhR^{ko} augmentaient significativement lors du stress oxydatif induit par l'extrait de fumée de cigarette (CSE) par rapport au type sauvage. De même, nous avons constaté une diminution de la viabilité cellulaire dans les cellules déficientes en AhR après traitement par le CSE, par rapport au type sauvage. L'expression de l'ARNm des gènes antioxydants majeurs Nqo1 et Srxn1 a également été fortement augmentée dans les cellules $AhR^{+/+}$, avec une diminution significative de l'induction dans les fibroblastes $AhR^{-/-}$. Afin de déterminer la manière dont Srxn1 et Nqo1 sont régulés par l'AhR, nous avons utilisé des fibroblastes pulmonaires dérivés de souris exprimant un AhR incapable de se lier au DRE ($AhR^{DBD/DBD}$). Suite à l'exposition au CSE, les niveaux de Nqo1 se sont trouvés significativement abaissés pour les fibroblastes $AhR^{DBD/DBD}$ par rapport au type sauvage, tandis que l'expression de Srxn1 était régulée positivement dans les cellules $AhR^{DBD/DBD}$ et les

cellules de type sauvage. Ainsi, nos résultats indiquent que l'expression de Nqo1 est gouvernée par l'AhR et dépendant de sa liaison au DRE, tandis que celle de Srxn1 serait peut-être régulée de manière non-génomique. Ces résultats pourraient révéler un mécanisme antioxydant protecteur de l'AhR dans la défense contre le stress oxydatif induit par le CSE.

List of Abbreviations

3-MC – 3-methylcholanthrene

4HNE – 4-Hydroxynonenal

8-OHdG – 8-Hydroxydeoxyguanosine

AHH – Aryl hydrocarbon hydroxylase

αNF – α-Naphthoflavone

ARE – Antioxidant response element

ARNT – Aryl hydrocarbon receptor nuclear translocator

B[*a*]P – Benzo[α]pyrene

Bax – Bcl-2-associated X protein

Bcl-2 – B cell lymphoma-2

CH-223191 – 1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl) diazenyl] phenyl-1H-pyrazole-5-carboxamide

CLIP-seq – Cross-linking immunoprecipitation-sequencing

COPD – Chronic Obstructive Pulmonary Disease

COX-2 – Cyclooxygenase 2

CSE – Cigarette smoke extract

CYP1A1 – Cytochrome P450, family 1, subfamily A, polypeptide 1

DBD – DNA binding-deficient

DIM – Diindolylmethane

DNA – Deoxyribonucleic acid

DRE – Dioxin response element

ER – Estrogen receptor

FICZ - 6-Formylindolo(3,2-b)carbazole

GOLD – The Global Initiative for Chronic Obstructive Lung Disease

GPx – Glutathione peroxidase

GSH – Glutathione (reduced)

GSK-3 β - Glycogen synthase kinase-3β

GSSG – Glutathione (oxidized)

GST – Glutathione S-transferase

H_2DCFDA – 2'7'- Dichlorodihydrofluorescein

Hsp90 – Heat shock protein 90

HuR – Human antigen R

I3C – Indole-3-carbinol

IL-1β – Interleukin 1 beta

ITE – 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester

Keap1 – Kelch-like associated protein 1

LPS – Lipopolysaccharide

miRNA – micro RNA

MMP – Matrix metalloproteinase

mRNA – Messenger RNA (ribonucleic acid)

NAC – N-acetyl-L-cysteine

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NLS – Nuclear localization sequence

Nox – NADPH oxidase

Nqo1 – NAD(P)H quinone oxidoreductase 1

Nrf2 – Nuclear factor (erythroid-derived 2)- like 2

PAH – Polycyclic aromatic hydrocarbons

PARP – Poly ADP ribose polymerase

PAS – Per-ARNT-Sim receptor family

PCB – Polychlorinated biphenyl

Prx – Peroxiredoxin

RelA – Transcription factor p65

RelB – Transcription factor RelB

ROS – Reactive oxygen species

SFN – Sulforaphane

SOD – Superoxide dismutase

Srxn1 – Sulfiredoxin 1

TCDD – 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

TNFα – Tumor necrosis factor alpha

TR – Thioredoxin reductase (human gene)

TRx – Thioredoxin

Txnrd1 – Thioredoxin reductase (murine gene symbol)

XAP2 – Hepatitis B virus X-associated protein

ZFNs – Zinc finger nucleases

1. Introduction

1.1 Tobacco History and Epidemiology of Disease

Tobacco is a product of the *Nicotiana* genus of plants used by indigenous Americans in pre-Columbian times and later discovered by Europeans. Early explorers of the Caribbean in the 15th century observed the medicinal use of tobacco by the native population. It was claimed that powdered tobacco could be applied locally for pain, sniffed to relieve headaches, or smoked to relieve colds and fevers; these purported medicinal uses motivated sailors to bring seeds and plants to Europe (1). Conditions were ripe in Europe for the introduction of new medicines to treat diseases that lacked cures. The exoticism of substances brought back from the New World, coupled with an enthusiasm for stories of medical usage by indigenous people, primed *Nicotiana* for widespread application (2). In the Columbian era, it was believed by Europeans to be a new panacea for the treatment of wounds, burns, conditions of the liver, and various infectious diseases (2,3). The medical use of tobacco continued through the mid-19th century despite increasing distrust of its efficacy by physicians and scientists.

Tobacco is presently the number one cause of preventable mortality worldwide. In the 1990s, tobacco smoke accounted for 3 million deaths worldwide annually; by 2030 it is projected that this number will reach 10 million (4). In Canada, tobacco usage accounts for 16.6% of total deaths annually. Each day, 100 Canadians die of a cigarette smoke-related disease (5). Smoking is linked to increased incidence of cardiovascular disease, such as stroke and coronary heart disease, cancers of the lung, and respiratory illness such as chronic obstructive lung disease (COPD).

1.2 Chronic Obstructive Lung Disease

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) report of 2017 defines COPD as "a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases." (6) Airflow limitation in COPD is also characterized by a lack of reversibility of airflow restriction by pharmaceuticals, which distinguishes it from other obstructive lung disease such as asthma (7). Two classic features of COPD are bronchitis and emphysema (7). Bronchitis- inflammation of the small lung airways- leads to increased mucus production and epithelial remodeling, thereby causing airflow limitation. Similarly, emphysema-related airflow limitation results from airspace enlargement due to alveolar, epithelial, and parenchymal cell destruction (8). COPD, however, can exist as a syndrome of chronic airflow obstruction with patients experiencing either one of the pathologies, but not necessarily both. COPD is largely caused by cigarette smoking, with 80% of COPD-related mortalities linked to cigarette smoke exposure (9).

1.2.1 Tobacco Smoke Chemistry

Tobacco smoke contains more than four thousand compounds distributed between the tar and gas phase, both of which contain highly reactive chemicals linked with pathophysiology (10). These include polycyclic aromatic hydrocarbons (PAH), acrolein, nitrosamines, benzenes, quinones, as well as many others. Cigarette smoke also contains a significant quantity of reactive oxygen species (ROS) that induce oxidative stress (11–13). ROS are a class of molecules derived from molecular oxygen that are deprived of a complete electron pair. Highly reactive ROS can bind to

and damage cellular components such as DNA, mitochondria, proteins, and the lipid membrane (14) (See **Chapter 1.3** for a more detailed discussion of oxidative stress). Indeed, cigarette smoke and cigarette smoke-induced oxidative stress are key to understanding COPD pathogenesis.

1.2.2 Mediators of COPD Pathogenesis: Cytokines, Oxidative Stress, and Proteinases

The primary role of the immune system is defense from environmental stress and pathogens, and localized inflammation is used to clear toxins or combat pathogenic microbes. The introduction of cigarette smoke, however, can drive the system out of balance, provoking an excessive inflammatory response. Cigarette smoke damages the structural cells of the lung, including lung fibroblasts and epithelial cells, triggering leukocyte recruitment and activating inflammatory cytokine expression (15,16). In response to cigarette smoke, activated lung epithelial cells produce cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins including IL-1β and IL-8. Advanced cases of COPD are correlated with neutrophil infiltration of the airway epithelium, submucosa, and smooth muscle (17). Neutrophil recruitment further perpetuates the production of inflammatory mediators, exemplified by the heightened expression of leukocyte chemotactic factor CCL5 in COPD lungs compared to those of nonsmokers or healthy smokers (18). Recruitment of cytotoxic CD8+ T cells into the parenchyma of COPD lungs also contributes to airway inflammation and is positively correlated with disease severity (19).

Involvement of macrophages is central to cigarette smoke-induced lung inflammation and COPD. There is a robust induction (5-10 fold) of macrophage infiltration in airways, bronchial alveolar lavage, and sputum in COPD patients (20). In response to noxious material in cigarette smoke, macrophages produce inflammatory cytokines, ROS, and proteinases that contribute to disease progression. Expression of inflammatory cytokines in both lung structural cells and

lymphocytes is governed by the transcription factor nuclear factor-κB (NF-κB). Indeed, NF-κB is more highly activated in COPD lungs compared to healthy tissues (21). The heightened inflammation in COPD patients and experimental models for chronic lung disease amplify the effects of ROS present in both cigarette smoke and ROS released by activated leukocytes. Lung damage as a result of cigarette smoke-induced oxidative stress is further perpetuated by ROS-generated inflammatory cellular mediators (22).

A key factor contributing to the pathology of COPD is oxidative stress. As mentioned previously, cigarette smoke is rich in ROS, small reactive molecules often with unpaired electrons that damage cellular components (14). Leukocytes recruited to damaged lung tissues secrete ROS in response to inflammation, thus creating a self-sustaining feedback loop that perpetuates oxidative stress, a state of ROS-antioxidant imbalance hallmarked by damage to DNA, proteins, and lipids (23,24). COPD lungs exhibit increased markers for oxidative stress, including 8-OHdG (oxidized DNA), phosphorylated histone 2AX foci (indicating DNA double-strand breaks) and 4HNE (lipid peroxidation) (25–27). Oxidative stress-induced DNA and mitochondrial damage can lead to apoptosis, a key pathological feature of airspace enlargement (see **Chapter 1.2.3**).

In addition to inflammation and oxidative stress, a third mechanism for the destruction of lung tissues in emphysema is an imbalance of proteinases and antiproteases. Cigarette smoke-induced ROS and inflammatory cytokines can induce proteinase expression and secretion while directly inhibiting antiprotease activity or expression (28–30). Cigarette smoke-induced oxidation of the key antiprotease, α1-antitrypsin, leads to a robust decrease in enzymatic inhibition of secreted elastase by α1-antitrypsin (31,32). Thus, antiprotease deficiency increases structural cell damage by proteinases such as elastase or the matrix metalloproteinases (MMPs). MMP expression is

furthermore upregulated by cigarette smoke-induced oxidative stress, by cytokines present in heightened inflammatory lung conditions (*e.g.* TNF-α), and by NF-κB activation (33–35). Taken together, this pathophysiological triumvirate – inflammation, oxidative stress, and antiprotease imbalance – provides a mechanism for the induction of emphysema in COPD (30).

1.2.3 Apoptosis

Evidence suggests that apoptosis is a major feature of airspace enlargement in emphysema (8,36,37). Apoptosis, or programmed cell death, is a tightly regulated molecular process initiated by the cell under stressful conditions. Apoptosis is necessary for normophysiology in numerous situations, including development, cell turnover, and immune system regulation. Dysregulation of apoptosis has been implicated in disease etiology. In conditions such as cancer, for example, there is too little apoptosis. In contrast, apoptosis may occur in excess in degenerative diseases such as emphysema. Apoptosis occurs through two molecular processes, the extrinsic and intrinsic pathways. In the present work, the intrinsic pathway is particularly relevant.

The intrinsic pathway begins with a disruption in mitochondrial membrane potential. The BAX family of proteins senses mitochondrial health and executes the apoptotic cascade when mitochondrial membrane potential drops (38). Poor mitochondrial health can be instituted by ROS such as those found in cigarette smoke (39,40). The protein known as B cell lymphoma-2 (Bcl-2), which gates the mitochondria, is downregulated by Bcl-2-associated X protein (Bax), allowing cytochrome C to translocate from the mitochondria into the cytoplasmic space. The translocation of cytochrome C is a critical event that triggers the activation of caspase-9, further leading to caspase-3 activation. Caspase-3, coordinates the cleavage of poly ADP ribose polymerase (PARP), which then fragments DNA (41–43). Other mediators of apoptosis are induced to ensure a robust all-or-nothing

response. Cellular fragmentation, membrane blebbing, and shrinkage are morphological changes in apoptosis and allow for the easy clearance of apoptotic bodies by circulating macrophages, which phagocytose the remaining particles. Removal of apoptotic bodies is a necessary step, as the unregulated release of intracellular components from dying cells onto the surrounding area can trigger a strong inflammatory response. Indeed, numerous links have been established between respiratory disease and inefficient clearance of apoptotic cells in the lung (30,42–47).

The intrinsic apoptotic pathway has been implicated in cigarette smoke-related diseases. In response to cigarette smoke exposure, lungs from Wistar rats had a dose-dependent increase in cellular protein expression of Bax and cleaved caspase-3, and a decrease in Bcl-2 as well as released cytochrome C (39,44). Oxidative stress triggers mitochondrial dysfunction and intrinsic apoptosis, while ROS in cigarette smoke oxidize cytoprotective factors such as Bcl-2, leading to their inactivation and caspase activation (48). Furthermore, p53, a key sensor of apoptosis that transcribes for pro-apoptotic factors under stressful conditions, is activated by cigarette smoke (47). Activation of p53 ensures an all-or-nothing apoptotic response, thereby downregulating the mRNA expression of key cytoprotective factors that include Bcl-2 and various antioxidant genes (49,50). Antioxidant gene expression and regulation protects against apoptosis before the caspase cascade is activated (51–53). In addition, antioxidants are downregulated in COPD, implicating oxidative stress-induced apoptosis as an important pathophysiological component.

1.3 Reactive Oxygen Species and Antioxidant Defense

1.3.1 Cigarette Smoke-Induced Free Radical and Antioxidant Biochemistry

Free radicals are generally small molecules with an unpaired electron. This class of compounds includes oxygen-containing ROS which damage cells and tissues through various physical means (14). An excess ratio of ROS to cellular antioxidants causes oxidative stress. In this condition, the unpaired electron in ROS oxidizes the DNA base guanosine causing genetic lesions, and oxidizes functional groups on proteins in the cytosol, membrane lipids, endoplasmic reticulum, and mitochondria. Cigarette smoke is a major inducer of oxidative stress in the lung (11,12,37). If cellular repair mechanisms cannot compensate for the excess oxidative stress, then the cell undergoes apoptosis to limit damage to the organism as a whole. ROS production is associated with the pathogenesis of COPD as well as COPD co-morbidities such as insulin resistance (54–57).

The typical ROS found in cigarette smoke (Figure 1.1) include superoxide ($O2^{\cdot-}$) and the hydroxyl radical. To prevent damage to lung cells, a strong antioxidant or phase II detoxification system must be in place to quench or dispose of ROS. In experimental models in cells and animals, pretreatment with antioxidants including N-acetyl-L-cysteine (NAC) and pomegranate polyphenols can protect against apoptosis and airspace enlargement following cigarette smoke exposure (58,59,13). The induction of cellular antioxidants is central to cytoprotection against oxidative stress. A crucial component of inducible antioxidant pathways is glutathione (GSH), a tripeptide assembled from cysteine, glutamic acid, and glycine (60,61). Thiol groups present on the cysteinyl moiety react with oxidants and oxidized proteins to detoxify them. This reaction generates an oxidized dimer of GSH, known as GSSG, which in turn is reduced back to GSH by the NADPH-

dependent enzyme glutathione reductase. As will be shown, GSH participates in many important antioxidant detoxification pathways.

Free radical	Representation	Half-life (s)
Superoxide	$O2^{\bullet-}$	10^{-6}
Hydroxyl radical	•OH	10^{-9}
Hydrogen peroxide	H_2O_2	Stable
Peroxyl radical	RCOO•	Seconds
Organic hydroperoxide	RCOOH	Stable
Singlet oxygen	$^1O_2$	10^{-6}
Ozone	O_3	Seconds

Figure 1.1. Names of common ROS involved in cell biology, chemical representation, and respective half-lives. The above ROS are found in or generated from cigarette smoke. (From (62))

A classical pathway (Figure 1.2) for the detoxification of $O2^{\cdot-}$ begins with the enzyme superoxide dismutase (SOD). SOD exists in three forms: CuZnSOD is localized to the cytosol, MnSOD is localized in the mitochondria, and SOD3 is extracellular (63). These enzymes reduce $O2^{\cdot-}$ to hydrogen peroxide (H_2O_2); enzymes such as catalase and glutathione peroxidase (GPx) then convert H_2O_2 to water. Peroxiredoxin (Prx) also reduces H_2O_2 to water, resulting in the oxidation and inactivation of Prx. Thiols, ascorbic acid, thioredoxins (Trx) and sulfiredoxin (Srxn) reactivate hyperoxidized Prx through the reduction of its cysteinyl moieties. Together these enzymes

constitute a robust and pleiotropic antioxidant defense system to protect against oxidative stress-induced toxicity (64–66).

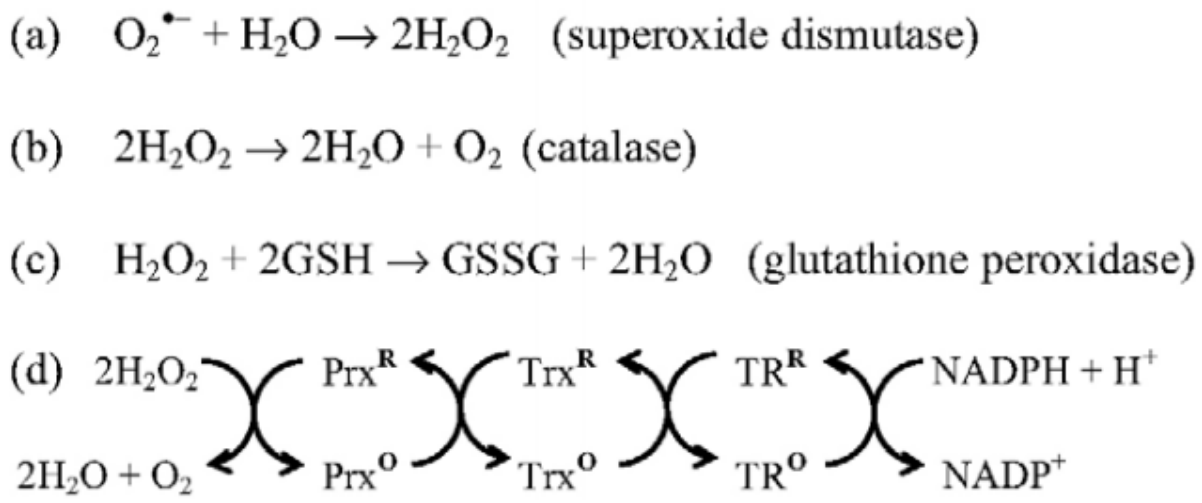


Figure 1.2. Cellular antioxidant pathways. **(a)** Detoxification of superoxide by superoxide dismutase. **(b)** Detoxification of hydrogen peroxide by catalase. **(c)** Detoxification of hydrogen peroxide by glutathione peroxidase, using the oxidation of the tripeptide antioxidant glutathione. **(d)** Detoxification of hydrogen peroxide with the NADPH-dependent peroxiredoxin/thioredoxin system (Adapted from (65)).

The pathway just described involves only $O_2^{\bullet-}$ and H_2O_2 and therefore is not applicable to all free radicals present in cigarette smoke. Semiquinones and acrolein, for example, cannot be chemically transformed into water. Instead, detoxification occurs through their excretion from the organism, facilitated by phase II metabolizing enzymes such as glutathione S-transferase (GST). GST conjugates GSH with hydrophobic xenobiotics and free radicals in order to neutralize their biological reactivity and transport them out of the cell and organism. The GST pathway is often accompanied by the addition of oxygen to xenobiotics in phase I metabolism (see **Chapter 1.5**). In

particular, GST detoxifies and protects against cigarette smoke. Studies indicate that single nucleotide polymorphisms in GSTP1, a subset of GST, increase risk for COPD development in humans (67). Additionally, overexpression of GSTP1 protects human lung fibroblasts from cigarette smoke extract-induced apoptosis *in vitro* (68). Together, these findings highlight the importance of phase II detoxification in antioxidant response against cigarette smoke-induced lung disease. It is noteworthy that there are numerous antioxidant and phase II enzymes relevant to COPD including glutathione peroxidases and glutaredoxins; for the sake of brevity, these are not included in this discussion.

1.3.2 The Transcriptional Antioxidant Response

Given the critical function of antioxidant and detoxifying enzymes in cellular survival, the expression of SOD and GST is regulated by many transcription factors such as activator protein-1 (AP-1), CCAAT-enhancer-binding protein (C/EBP), peroxisome proliferator-activated receptor γ (PPARγ), and specificity protein 1 (Sp1) (69). In addition, the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), sometimes regarded as "the master regulator of antioxidant response" (70), plays a significant role. Nrf2 is held in the cytoplasm by its repressor kelch-like associated protein 1 (Keap1), which ubiquitinates Nrf2 for proteosomic degradation. Upon oxidative insult, however, thiol groups on Keap1 are oxidized, liberating Nrf2 and allowing for its nuclear translocation. In the nucleus, Nrf2 binds to a specific sequence of DNA (RTGACnnnGC) known as the antioxidant response element (ARE), present in the promoter regions of genes that code for antioxidant and phase II detoxifying enzymes (70–72,66). Figure 1.3 shows genes regulated through Nrf2-ARE-mediated activity.

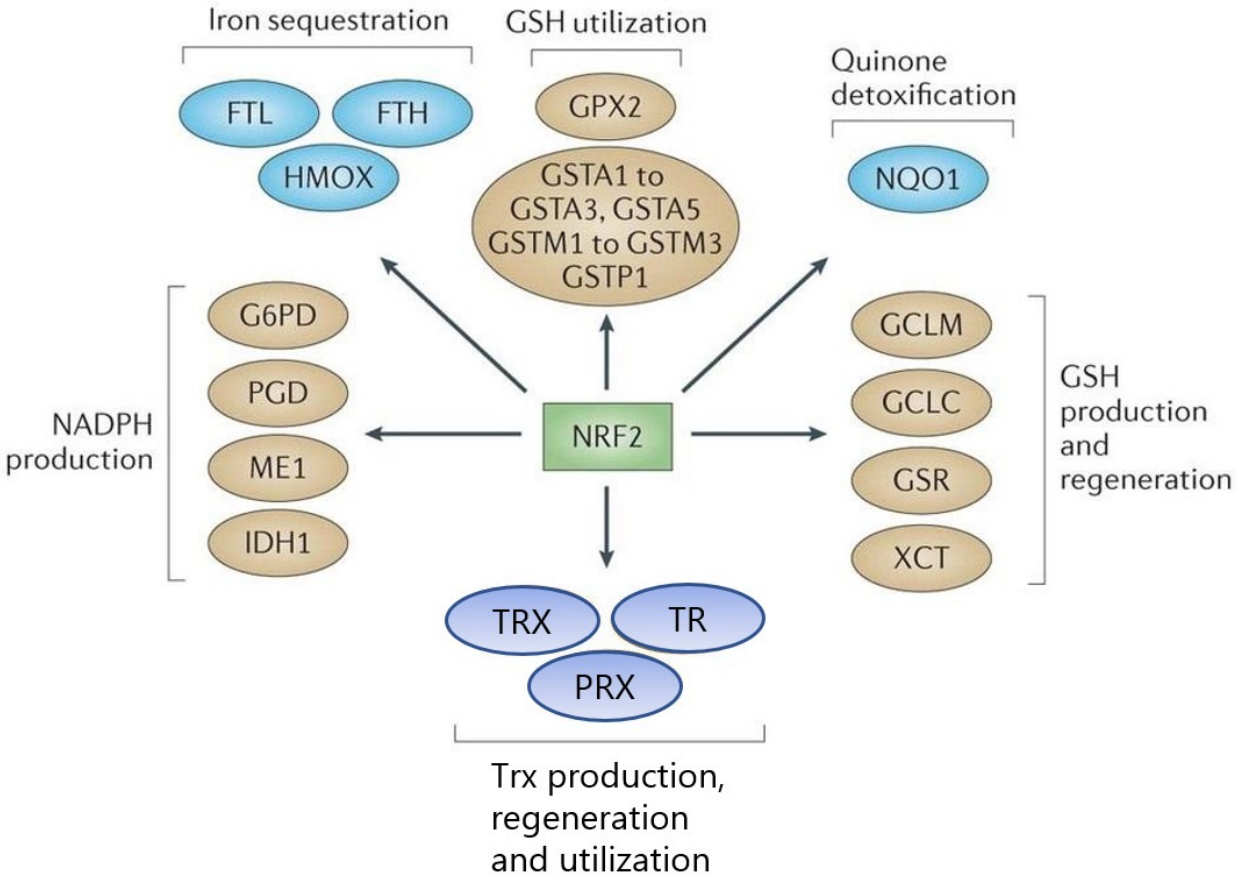


Figure 1.3. Nrf2 activation and binding to the ARE induces a gene battery that transcribes for phase II detoxifying enzymes involved in glutathione production and utilization (e.g. GPX, GST genes), quinone detoxification (NQO1) and peroxiredoxin pathway function (TRX, PRX) among others (Adapted from (70)).

As cigarette smoke exposure introduces a strong oxidative insult to lung cells, Nrf2 activity serves a cytoprotective role. It has been theorized that genetic deficiency in Nrf2 activity or expression could provide a mechanism for predisposition to cigarette smoke-induced lung disease such as COPD. Interestingly, genetic ablation of Nrf2 increases sensitivity to cigarette smoke-induced emphysema in mice (73). Neutrophilic lung inflammation in Nrf2-knockout mice was significantly higher compared to wild-type mice after 8-16 weeks of cigarette smoke exposure.

Nrf2-knockout mice had a significant imbalance of anti-proteases to proteases, leading to pathological tissue destruction typical of emphysema. In a separate study, Nrf2-deficient mice treated for 6 months with cigarette smoke had a robust increase in the oxidative stress marker 8-OHdG and alveolar apoptosis compared to wild-type (74). Numerous cell culture studies further implicate Nrf2 in protection against cigarette smoke-induced oxidative stress and apoptosis (75–78). Genetic alterations in Nrf2 have been investigated as a risk factor for COPD, although evidence thus far has been inconclusive (79–81). Nonetheless, Nrf2 has been examined as a drug target for COPD. Dietary constituents that activate Nrf2, such as sulforaphane, are being investigated for their efficacy in treating COPD (65,82).

The importance of Nrf2 in antioxidant gene regulation is evident. Newer research, however, has indicated that the aryl hydrocarbon receptor (AhR) may also have antioxidant and anti-apoptotic effects against cigarette smoke-exposed fibroblasts independent of Nrf2. Additional mechanisms, such as those involving the AhR, may also be involved in protection against cigarette smoke, as discussed in the following section.

1.4 The Aryl Hydrocarbon Receptor

The AhR is a ubiquitously expressed cytosolic transcription factor best understood for its role in mediating the toxic effects of environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polycyclic aromatic hydrocarbons (PAH) including benzo[α]pyrene (B[*a*]P), and polychlorinated biphenyls (PCBs). Although it is presently recognized that AhR has endogenous roles in physiology, for the last 30 years it has been described in a purely

toxicological context. The toxic effects of halogenated aromatic hydrocarbons (more simply, dioxins and dioxin-like compounds) include developmental, immunotoxic, neurotoxic, and carcinogenic activities (83,84). Dioxins are unintentionally produced in industrial settings such as paper mills or metal smelters and accidentally released into the environment via landfill or as vapor from trash combustion. Between 1930 and 1976, over 1.3 billion pounds of dioxins were produced, 1.25 billion pounds of these by the United States agricultural and chemical producer, Monsanto (85). The use of the TCDD-containing defoliant Agent Orange in the Vietnam War represented one of the most challenging humanitarian and environmental catastrophes of the era. Soldiers and civilians alike were victims of TCDD exposure from Agent Orange, resulting in various types of cancers as well as neurodegenerative and metabolic disease (86–89). Throughout the 20th century, incidents of accidental contamination of food products with dioxin-like compounds have resulted in morbidity for humans (90–92).

Early research indicated that exposure of PAH fed to young female rats would lead to mammary carcinoma within a few weeks (93,94). At that time the carcinogenic effects of such compounds were thought to result from interference with hormonal systems involving estrogen, given the structural resemblance of PAH to endogenous steroids. Researchers began to hypothesize that PAH could be targeting a unique receptor for activation. This recognition was partially based on early work that showed that PAH could induce the expression of a drug-metabolizing enzyme, known at the time as aryl hydrocarbon hydroxylase (AHH) and presently as cytochrome P450 1A1 (CYP1A1). In 1969 Nebert, et al. demonstrated that mice of various genetic lineages displayed differential induction of AHH by B[*a*]P and 3-methylcholanthrene (3-MC), with some strains having no observable effect (95,96). In the mid-1970s, when public awareness of TCDD was at its peak, Alan Poland at the University of Rochester found that TCDD induced robust expression of

AHH in chick embryo liver. Adding to this finding, TCDD was found to induce AHH expression 3000-fold greater than 3-MC (97). Together with Nebert, Poland showed that TCDD could induce AHH expression in mice insensitive to induction by B[*a*]P or 3-MC (98). These findings shifted the focus of research away from AHH enzymatic activity toward its upstream regulation. Using radiolabeled TCDD in conjunction with classic enzymatic experimental techniques, the elusive regulator of AHH – *i.e.*, the AhR – was discovered (99).

Since its initial discovery, research into AhR has shifted from its toxicological role toward defining endogenous roles for this receptor. After all, one may ask, why would evolutionary selection pressure result in a receptor that mediates toxicological outcomes to anthropogenic chemicals from the last 100 years? It is likely that a separate, endogenous role for the AhR was evolved that scientists have yet to fully elucidate. This idea is supported by evidence that only AhR expressed in vertebrates produce toxicological responses to dioxin. AhR orthologues from nematode (C. elegans), fruit fly (D. melanogaster), and soft-shell clam (M. arenaria) do not bind to TCDD and instead may be involved with neuronal development (100–103). The 1990s saw the identification of a binding partner for AhR, the aryl hydrocarbon receptor nuclear translocator (ARNT), as well as the classification of AhR as a member of the Per-ARNT-Sim (PAS) receptor family (104,105). Additionally, putative endogenous ligands for the AhR were discovered, such as 6-formylindolo[3,2-b]carbazole (FICZ), which is formed from UV-catalyzed metabolism of L-tryptophan (106). A recent renaissance in AhR research continues to reveal novel endogenous roles for this receptor protein, underscoring its importance in various cellular processes.

1.4.1 The Canonical Pathway of the AhR

The AhR is held in the cytosol by chaperone proteins, which include two copies of heat shock protein 90 (Hsp90) as well as hepatitis B virus X-associated protein (XAP2) and p23. Upon binding to a ligand, the AhR dissociates from these chaperones and translocates to the nucleus, binding to ARNT. The AhR-ARNT dimer binds to a sequence of DNA (5'-TNGCGTG-3') known as the dioxin response element (DRE) found in the promoter sequences of genes involved in xenobiotic metabolism such as CYP1A1 and glutathione S-transferase (Figure 1.4) (107,108). This classic pathway of AhR-mediated transcription is also regulated by recently discovered endogenous ligands.

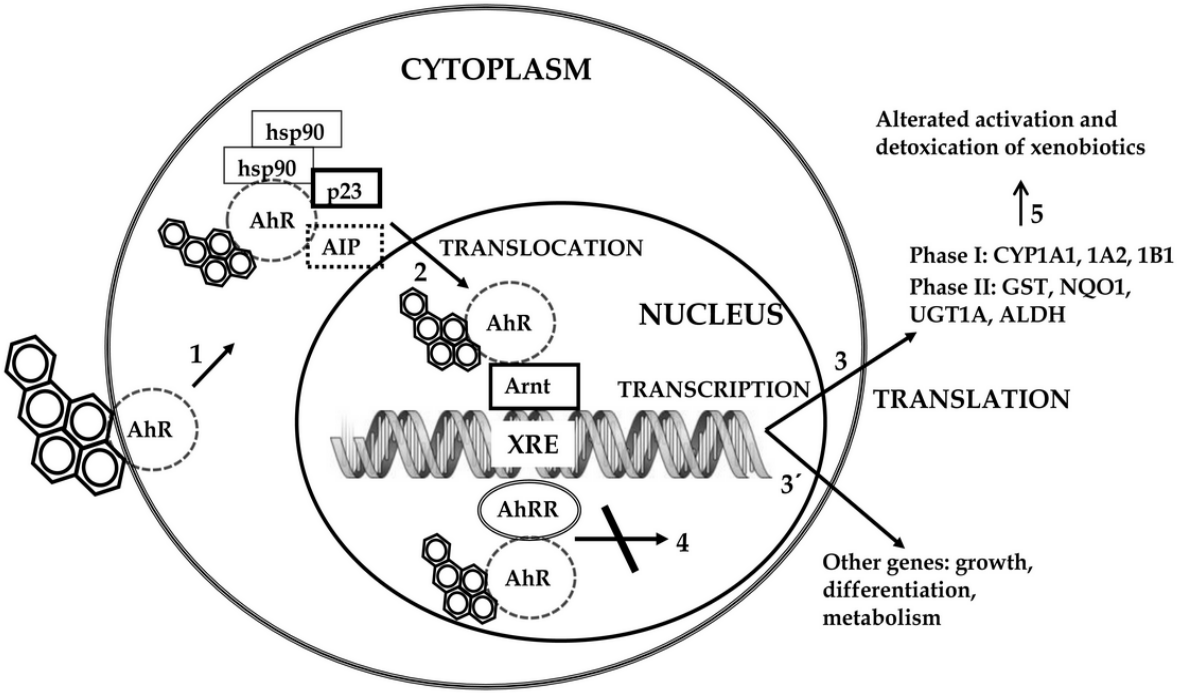


Figure 1.4. Ligand-dependent AhR activity results in its disassociation from cytosolic chaperones including Hsp90, p23, and XAP2 (also known as AhR-interacting protein, AIP) and translocation to the nucleus. After partnering with ARNT, the receptor complex binds to DNA at xenobiotic (or dioxin) response element sites (XRE, or DRE) in genes coding for phase I and II detoxification enzymes. AhR signaling is terminated by the aryl hydrocarbon receptor repressor (AhRR). (From (109))

1.4.2 Ligands of the AhR

Unlike dioxins, the tryptophan metabolites FICZ and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) activate the AhR without promoting a toxic, dioxin-like effect (110,111). Activation of the AhR by these compounds, however, promotes robust physiological effects, especially on the regulation of experimental autoimmune conditions in rodents. ITE, for example, reduces experimental autoimmune uveitis by downregulating Th1 and Th17 cytokines in an AhR-dependent manner (110). Numerous dietary chemical constituents also ligate the AhR, including diindolylmethane (DIM). DIM is formed in the gut from indole-3-carbinol, which naturally occurs in cruciferous vegetables (Figure 1.5) (112). Other ligands such as indirubin activate the AhR in a nontoxic manner (113,114). Indirubin is present in the traditional European herbal remedy woad (*Isatis tinctoria*) and has been used for centuries for the treatment of inflammation (115).

One reason why some classic exogenous AhR ligands produce toxic effects may be because of their bioactivation by AhR-mediated transcriptional activity. B[*a*]P, a typical polycyclic aromatic hydrocarbon produced from the incomplete combustion of organic material, induces AhR activation and transcription of CYP1A1 (116,117). CYP1A1, a key inducible monooxygenase, adds polar handles to lipophilic compounds, thereby rendering them water soluble and facilitating their excretion from the cell and organism. Due to the aromaticity of B[*a*]P, however, CYP1A1-mediated addition of oxygen results in the formation of a reactive epoxide that can directly damage DNA (118). In excess, this epoxide can cause sufficient DNA damage to transform the cell or induce apoptosis. On the other hand, dioxins such as TCDD induce DNA damage by virtue of activating AhR-mediated cytochrome P450 so strongly that oxidative stress occurs (119). Although the

aforementioned could explain the means by which TCDD-induced apoptosis occurs, the precise mechanism is not yet understood (120).

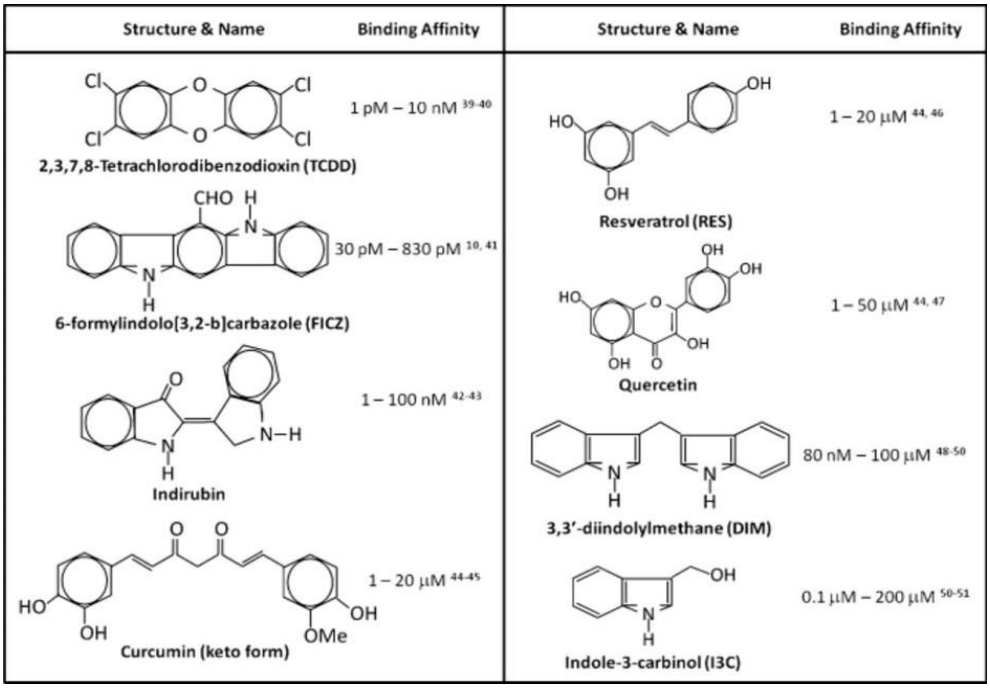


Figure 1.5. Common endogenous and exogenous ligands of the AhR. Although TCDD is the prototypical environmental AhR ligand, dietary xenobiotic ligands for the AhR such as curcumin, resveratrol, quercetin, and diindolylmethane also activate the AhR, albeit with lower affinity. FICZ, a tryptophan metabolite, is one of the principal endogenous AhR activators. Adapted from Busbee, et al. 2013. (From (112))

1.4.3 The Canonical Pathway of the AhR in Development

In response to TCDD, activation of the AhR results in tumor promotion, hepatocellular damage, immune suppression, and thymic involution (121). This effect is absent in TCDD-treated homozygous *Ahr* null mice. Interestingly, however, *Ahr* null mice display developmental defects including a patent ductus venosus, resulting in significantly reduced liver weight characterized by paleness, sponginess, and fibrosis (122). Hepatic lipid production is also disrupted. Together, these findings indicate that although the AhR mediates toxic outcomes of dioxin exposure, its expression is necessary for healthy liver development. Furthermore, mice lacking the AhR nuclear localization sequence (Ahr^{nls}) or with DNA binding-deficient AhR genotypes ($AhR^{DBD/DBD}$) displayed developmental liver defects similar to developmental defects observed in *Ahr* null mice (123–125). Tolerance to TCDD-induced toxicity was also found in these transgenic mice. Hence, it appears that AhR nuclear translocation and DNA binding are crucial for both developmental processes and toxicological outcomes.

1.4.4 Noncanonical and Nongenomic Roles for the Aryl Hydrocarbon Receptor: Physical Interactions

Novel research on noncanonical and nongenomic roles for the AhR in cell signaling, homeostasis, and immune regulation paint a broader picture of putative endogenous activities for the AhR. Many of these activities may be due to cross-talk with other transcription factors and/or intracellular proteins. For example, the AhR interacts with and influences the activity of the estrogen receptor (ER). The ER has been shown to associate with the AhR as assayed by

coimmunoprecipitation *in vitro*. This association can occur as a result of treatment with both AhR ligand (*e.g.* TCDD, 3-MC) or ER ligand (estradiol, E2) (126,127). The AhR can attenuate the transcriptional response of target genes containing both DRE and ERE (estrogen response element) of the ER in response to E2 treatment. In this way, the AhR effectively fine-tunes estrogenic transcriptional activity. Additionally, in ovariectomized mice, the AhR promoted an estrogenic response to TCDD, an effect that was lost in both AhR knockout ($AhR^{-/-}$) and ER knockout mice. (127) Activation of the AhR by 3-MC also leads to increased ubiquitin-mediated proteasomal degradation of ER (128–130). A plasmid construct for the AhR expressing constitutive transcriptional activity without a ligand binding domain was employed to explore possible ligand-mediated effects of the AhR on proteasomal degradation of ER. It was found that regardless of ligand, constitutive AhR activity was sufficient to mediate the ubiquitination and degradation of ER (131). Paradoxically, the AhR appears to exert both estrogenic and anti-estrogenic properties, varying from one model to another, sometimes in a ligand- and dose-dependent manner. Although the exact relationship between the AhR and the ER is still under exploration, the working hypothesis for their association underlies a mechanism for how TCDD and other AhR-activating environmental contaminants disrupt endocrine activity. For example, it is known that there is an increased incidence of breast cancer among women in whom PAH are found in their adipose tissue. (132)

There is also cross-talk between the AhR and NF-κB subunits RelA and RelB. In both non-malignant and malignant human breast cancer cell lines, physical interaction between the AhR and RelA leads to binding of NF-κB DNA elements and transactivation of the oncogene *c-myc*, which mediates cellular proliferation (133). The positive correlation between the AhR and RelA also contributes to TCDD induced IL-6 production in H1355 human lung adenocarcinoma cells (134). TCDD exposure led to the increased recruitment of a complex of RelA with the NF-κB partner p50

to the κB element of the IL-6 gene promoter and enhanced IL-6 expression. Additionally, AhR overexpression led to enhanced formation of nuclear RelA/AhR complexes and increased NF-κB transcriptional activity without p50. RNA interference of AhR led to decreased IL-6 expression and a modulating role for the AhR in RelA activity (134). Additionally, AhR expression is mediated by RelA activation. Lipopolysaccharide (LPS), a component of gram-negative bacteria cell walls and elicitor of strong NF-κB-mediated immune responses in mammals, induced RelA binding to NF-κB elements present in the promoter region of the AhR and subsequent transcription (135).

The AhR also associates with the noncanonical NF-κB subunit RelB. In response to TCDD, RelB mediates expression of the proinflammatory cytokine IL-8, an effect that is dependent on the presence of the AhR (136). It was discovered that the AhR physically binds to RelB and is directed to a novel DNA response element, the RelBAhRE, in addition to the DRE (137). Furthermore, *AhR*$^{-/-}$ mice are significantly prone to heightened markers of inflammation– TNF-α and IL-6– in association with decreased RelB protein expression (138). Taken together, these data suggest that the AhR:RelB complex may constitute a novel cellular mechanism to moderate the inflammatory response.

1.4.5 Noncanonical and Nongenomic Roles for the Aryl Hydrocarbon Receptor: Post-Transcriptional Regulation

Aside from physical interactions between the AhR and other transcription factors impacting nongenomic activities, the AhR regulates microRNA (miRNA) levels. miRNA are small noncoding RNA fragments transcribed from DNA. After pre-miRNA is exported from the nucleus and processed by the ribonuclease DICER, mature miRNA binds to complementary messenger RNA

transcripts. This binding subsequently inhibits protein translation from the mRNA transcripts by causing mRNA cleavage or inhibiting translation. A novel role for AhR has been proposed in governing the expression of miRNA. *AhR*$^{-/-}$ mouse lung fibroblasts have significantly less microRNA 196a (miR-196a) compared to wild-type, a feature that contributes to enhanced apoptosis caused by cigarette smoke (139).

The AhR also controls the cellular localization of human antigen R (HuR). HuR is an RNA binding protein that shuttles transcripts into the cytosol, where it stabilizes target mRNA for protein translation. In response to cigarette smoke extract, the AhR inhibits the shuttling of HuR to the cytoplasm from the nucleus in mouse lung fibroblasts. HuR, which can stabilize mRNA transcripts for inflammatory proteins such as COX-2, was translocated to the cytoplasm following CSE exposure in *AhR*$^{-/-}$, but not wild-type, mouse lung fibroblasts. This effect was not dependent on binding of the AhR to the DRE (140). These studies represent important contributions to our understanding of DRE-independent functions for AhR against inflammation associated with cigarette smoke exposure. There is currently no information about a DRE-independent AhR pathway in the regulation of smoke-induced oxidative stress.

1.5 Hypothesis

I hypothesize that the AhR reduces oxidative stress through the regulation of antioxidant enzymes (e.g. Nqo1, Srxn1) in a DRE-dependent manner.

1.6 Aims

The aims of the present work are as follows.

Aim 1: Determine if AhR attenuates CS-induced oxidative stress in mouse lung fibroblasts.

Aim 2: Determine the molecular mechanism by which the AhR controls oxidative stress by examining AhR transcriptional activity of antioxidant gene mRNA.

2. Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. 2'7,'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes® (Eugene, OR). The competitive AhR antagonist (125) CH-223191 (1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl) diazenyl] phenyl-1H-pyrazole-5-carboxamide) was from Tocris Bioscience (Minneapolis, MN) and was used at a concentration of 10 μM (120,121).

2.2 Lung fibroblast culture

Mouse lung fibroblasts: Lung fibroblasts were obtained from $AhR^{+/-}$ and $AhR^{-/-}$ C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) using an established tissue explant technique (144). Lung fibroblasts were also generated from a novel lineage of mice harboring a mutant AhR that is incapable of binding to DNA ($AhR^{DBD/DBD}$) (124), a kind gift of Dr. Chris Bradfield (University of Wisconsin); lung fibroblasts from littermate heterozygotes ($AhR^{DBD/B6}$) were used as corresponding controls. $AhR^{+/+}$ and $AhR^{+/-}$ fibroblasts show no significant difference in response to cigarette smoke or classic AhR ligands and are therefore used interchangeably as AhR-expressing cells (140,145).

2.3 Generation of A549-AhR^{ko} cells

A549 human lung adenocarcinoma cells and an A549 strain deficient in AhR expression (hereafter referred to as A549-AhR^{ko}) were used in this study. Generation of A549-AhRko cells was

accomplished by zinc finger nucleases (ZFNs) and were a kind gift from Dr. Jason Matthews (University of Toronto). Briefly, 2 x 10^6 A549 cells were transfected with 2 μg of each ZFN plasmid targeting AhR using nucleofector kit V and Amaxa nucleofector (Lonza, Mapleton, IL) according to the manufacturer's recommendations. Three days after transfection, cells were serially diluted into 2 × 96-well plates from an initial seeding density of 100,000 cells/well. At least 24 clones generated from polyclonal cell populations that were screened for the presence of indels at the ZFN recognition site in exon 1 of AhR by DNA sequencing. AhR protein levels were assessed by immunoblotting. TCDD was used to induce *Cyp1a1* and *Cyp1b1* mRNA with low expressed levels indicating cell clones containing genetic alterations that resulted in out-of-frame mutations in AhR transcription.

2.4 Preparation of Cigarette Smoke Extract (CSE)

Research grade cigarettes (3R4F) with a filter were obtained from the Kentucky Tobacco Research Council (Lexington, KT). CSE was prepared by bubbling smoke from two cigarettes into 20 ml of serum-free MEM, the pH adjusted to 7.4, sterile- filtered with a 0.45-mm filter (Pall Corp., Ann Arbor, MI) and used within 30 minutes of preparation. An optical density of 0.65 (320 nm) was considered to represent 100% CSE and was further diluted to the appropriate concentration in serum-free MEM to between 2-10%. These concentrations have previously been shown by to induce apoptosis in primary lung fibroblasts (48,37).

2.5 Immunocytochemical imaging for Hoechst fluorescence

Increased fluorescence of the DNA dye Hoechst is indicative of chromatin condensation, a characteristic of cells undergoing apoptosis (139,146). Viable and apoptotic cells were counted and results were quantitatively expressed as the percentage of apoptotic cells compared with the total number of cells. $AhR^{+/-}$ and $AhR^{-/-}$ fibroblasts were cultured on glass chamber slides and treated with control media or with CSE for 6 hours. In separate experiments, cells were pre-treated with 1mM N-acetyl-L-cysteine (NAC) for 1 hour followed by exposure to CSE. After this, cells were fixed in methanol, incubated with the Hoechst stain for 15 minutes, cover-slipped and viewed Olympus BX51 microscope (Markham, ON). Photographs were taken using a QImaging® Retiga-2000R camera at 40x magnification and analyzed with Image-Pro Plus v. 7.0.

2.6 Real-Time-PCR (qPCR) Array

Detection and quantification of gene expression between CSE-exposed $AhR^{-/-}$ and $AhR^{+/+}$ fibroblasts was first performed by qPCR array. After exposure to 2% CSE for 6 hours, total RNA was isolated using the Qiagen miRNeasy® Mini Kit according to the manufacturer's instructions (Qiagen, Toronto, ON). RNA quality and quantity were assessed using a Nanodrop spectrophotometer and 250 ng of total RNA was reverse transcribed using SuperScript III (Invitrogen). The expression of approximately 84 genes was analyzed by qPCR using a commercial PCR array for oxidative stress (Oxidative Stress Array PAMM-014, SA Biosciences) according to the manufacturer's instructions. The relative level of mRNA expression for each gene in each sample was first normalized to the expression of two housekeeping genes (β-actin and GAPDH).

Values represent fold-regulation between media- and CSE-exposed cells based on AhR expression of those genes having a greater than 3-fold change in relative expression levels.

2.7 qRT-PCR for gene validation

RNA was harvested using 700µl of QIAzol® lysis reagent (Qiagen, Valencia, CA). Total RNA was extracted using miRNeasy Mini Kit (Qiagen) and diluted to a concentration of 5ng/µl in RNase-free water. cDNA was generated through reverse transcription using iScript IITM Reverse Transcription Supermix (Bio-Rad Laboratories, Mississauga, Ontario). The reaction was carried out at 25°C for 5 minutes followed by 42°C for 30 minutes. Real-time qPCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad). Amplification occurred in SsofastTM Eva Green® Supermix (Bio-Rad) with 1µl of cDNA and 0.5 µM of primers (Table 2.1). Amplification cycles were preceded by 3 minutes at 95°C, followed by 40 cycles alternating between denaturation for 5 seconds at 95°C and annealing for 5 seconds. Analysis of gene expression was performed using the ΔΔCt method, normalizing Ct values to a housekeeping gene (β-actin).

Table 2.1. Primer sequences utilized for qRT-PCR.

mRNA	Primer
Mus Musculus **Nqo1**	Sense: 5'-GCGGCTCCATGTACTCTCTTCA-3'
	Anti-sense: 5'-ACGGTTTCCAGACGTTTCTTCC-3'
Mus Musculus **Nrf2**	Sense: 5'-ATACGCAGGAGAGGTAAGAATAAAGTC-3'
	Anti-sense: 5'-AGAGAGTATTCACTGGGAGAGTAAGG-3'
Mus Musculus **Sod1**	Sense: 5'-AGCGGTGAACCAGTTGTGTTGT-3'
	Anti-sense: 5'-CGTCCTTTCCAGCAGTCACATT-3'
Mus Musculus **Sod2**	Sense: 5'-TTCTTTGGCTCATTGGGTCCT T-3'
	Anti-sense: 5'-GATAAACAGGGGCTTCGCTGAT-3'
Mus Musculus **Srxn1**	Sense: 5'-CTATGCCACACAGAGACCATAG-3'
	Anti-sense: 5'-GTTGACCTGCTAATGTGCTTTC-3'

2.8 Measurement of reactive oxygen species and apoptosis by flow cytometry

Oxidative stress was measured in *AhR*$^{+/-}$ and *AhR*$^{-/-}$ lung fibroblasts with CSE by using 5-(-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), a cell-permeant indicator for reactive oxygen species (ROS) that is nonfluorescent until oxidation occurs within the cell (37,147). Flow cytometric analysis of apoptosis also included changes in cell size and granularity that were determined by forward and side scatter profiles (37). For these experiments, equivalent numbers of cells were grown to confluence in 25-cm^2 cell culture flasks or 6 well plates, serum starved for 24 h, and then treated with 2% CSE for the pulmonary fibroblasts or 5% and 10% CSE for A549 cells, for 4 hours. Controls included incubation with serum-free medium alone with and without H_2DCFDA. After treatment cells were washed with PBS and H_2DCFDA (10 μM) was added for 20 min at 37°C. Cells were then trypsinized, washed, and resuspended in PBS. Flow cytometric analysis was performed with a Becton-Dickinson FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). A minimum of 1000 events were acquired for murine pulmonary fibroblasts and 10000 events acquired for A549 for each sample. Debris gated out and analysis for ROS production was examined in the viable cell population as determined by forward and side scatter plot distribution.

2.9. Western blot

Total cellular protein was prepared using Ripa buffer and 5–10 mg of protein were electrophoresed on polyacrylamide gels and electrotransferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Antibodies against AhR (1:5000; Enzo Life) and total Actin (1:10,000; Millipore, Temecula, CA) were used to assess changes in relative expression. Proteins were

visualized using HRP-conjugated secondary antibodies (1:50,000) followed by enhanced chemiluminescence (ECL) and imaged using a ChemiDoc XRSþ System (Bio-Rad).

2.10. Nrf2 luciferase assay

BEAS-2B cells were stably transfected (using the pGL4.28 vector) to express a luciferase reporter that has four antioxidant response elements (ARE) cloned from a gene highly regulated by Nrf2 (NQO1). Once generated, these cells were cultured in 10% fetal bovine serum with 100 U/mL penicillin G, 100 mg/mL streptomycin, and 200 µg/mL hygromycin. Following stimulation with 2% CSE or sulforaphane (SFN; 5 µM; Sigma), cells were washed with phosphate-buffered saline and 50 µL of reporter lysis buffer (Promega, Madison, WI) was added per well. Cells were scraped and spun down at 13,000 g for 3 min. Samples of 10 µL were used in 96-well plates for the reporter assay. A total of 30 µL of luciferase assay reagent (20mM Tricine, 1.07mM $(MgCO_3)$●4 Mg(OH) 2●5H_2O, 2.67mM $MgSO_4$, 0.1mM ethylenediaminetetraacetic acid, 33mM dithiothreitol, 270 µM coenzyme A, 0.477mM D-luciferin, and 0.533mM adenosine triphosphate) was added to each well using an automatic injector. Emission units were read on a Tecan Infinite M1000 plate reader.

2.11. Immunocytochemistry

$A549^{Parent}$ and $A549\text{-}AhR^{ko}$ cells were cultured on glass chamber slides and left untreated or were treated with 5% CSE for 4h (148). Following treatment, cells were washed once with PBS/ Tween, permeabilized/fixed using 3% H_2O_2/methanol for 10 min, and blocked with Universal Blocking Solution for 1 h at room temperature. The antibody against Nrf2 (Santa Cruz, 1:200) was

diluted in Antibody Diluent Solution (Dako) and incubated overnight at 4 °C. Alexa Fluor-555 anti-rabbit IgG antibody was used for secondary binding (1:1000) and incubated for 1 h at room temperature. Slides were then mounted in ProLong Gold Anti-Fade (Invitrogen), viewed on an Olympus IX71 fluorescent microscope (Olympus, Ontario, Canada), and photographed using a Retiga 2000 R camera with ImagePro Plus software. Fluorescent images of nuclei are visualized by Hoechst staining (1:2000). All procedures were performed at the same time to minimize variability in fluorescence intensity.

2.12. Nrf2 knockdown in primary cells

AhR$^{+/-}$ lung fibroblasts were seeded at 1–2 × 10^4 cells/cm^2 and transfected with 40 nM of siRNA against Nrf2 or nontargeting control siRNA according to the manufacturer's instructions. On the next day, cells were treated with 2% CSE and RNA or protein was collected for further analysis as described above. Verification of Nrf2 knockdown was done by qRT-PCR and western blot.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (v.6.02; La Jolla, CA). A two-way analysis of variance (ANOVA), followed by a Newman–Keuls multiple comparisons test, was used to assess differences between groups defined by two variables. Results are expressed as mean ± SEM. In all cases, a p-value < 0.05 is considered statistically significant.

3. Results

3.1 Expression of the AhR protects against cigarette smoke-induced cell death by attenuating reactive oxygen species (ROS) production

Cigarette smoke causes oxidative stress and apoptosis in lung structural cells (37). It has been previously shown that primary pulmonary fibroblasts derived from *AhR*$^{-/-}$ mice are more sensitive to the toxic effects of cigarette smoke, rapidly undergoing a significant decrease in viability when exposed to 2% CSE (48). To now determine whether AhR expression reduces ROS production in response to cigarette smoke, pulmonary fibroblasts, and A549 epithelial cells were treated with CSE for 4 hours and processed for flow cytometry. Our data show that in the absence of AhR expression, CSE induced a robust and significant increase in H_2DCFDA fluorescence intensity, indicative of heightened ROS production in both primary lung fibroblasts (Fig. 3.1A and 3.1B) and A549 epithelial cells (Fig. 3.1C and 3.1D).

To now determine the contribution of oxidative stress to the susceptibility of cigarette smoke-induced apoptosis, we pretreated *AhR*$^{+/+}$ and *AhR*$^{-/-}$ fibroblasts with NAC, an antioxidant and a precursor to GSH synthesis (37,149), prior CSE exposure and analyzed chromatin condensation as a marker of cell death (48,139). We found that NAC pretreatment abrogated chromatin condensation (as indicated by compact, brightly-fluorescent nuclei) that was increased in *AhR*$^{-/-}$ fibroblasts treated with 2% CSE (Fig 3.2A, arrowheads). By comparison, slight changes in chromatin condensation elicited by 2% CSE in *AhR*$^{+/+}$ fibroblasts (oval nuclei with minimal fluorescence) were unaffected by NAC pretreatment (Fig. 3.2A, open arrows). Quantification of fluorescent nuclei revealed that there was a significant reduction in the percentage of compact brightly-fluorescent nuclei when AhR$^{-/-}$ fibroblasts were treated with NAC in conjunction with 2%

CSE (Fig. 3.2B). When considered together, these findings suggest that the AhR plays a protective role against oxidative stress induced by cigarette smoke to enhance cell survival.

Figure 3.1

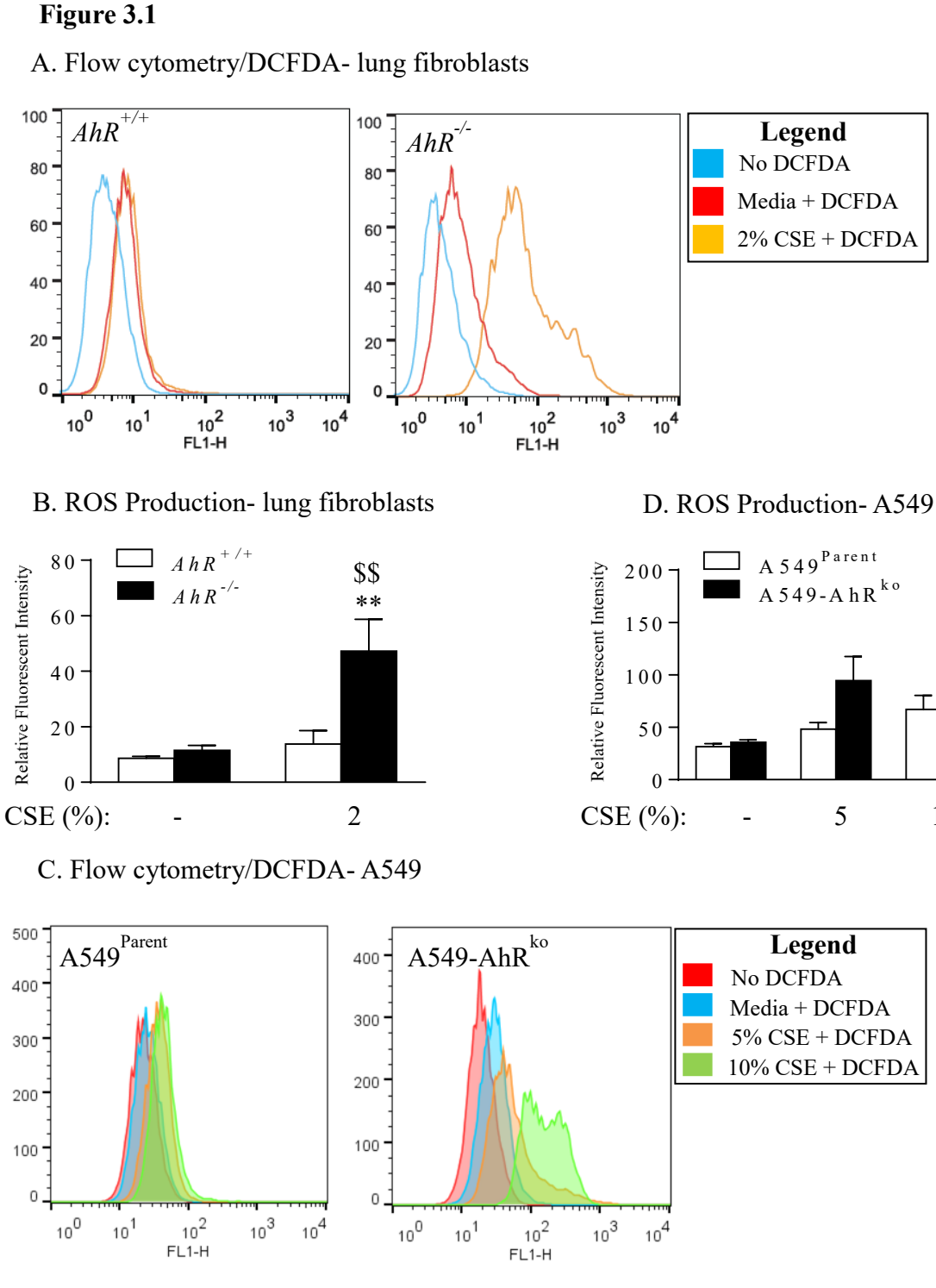
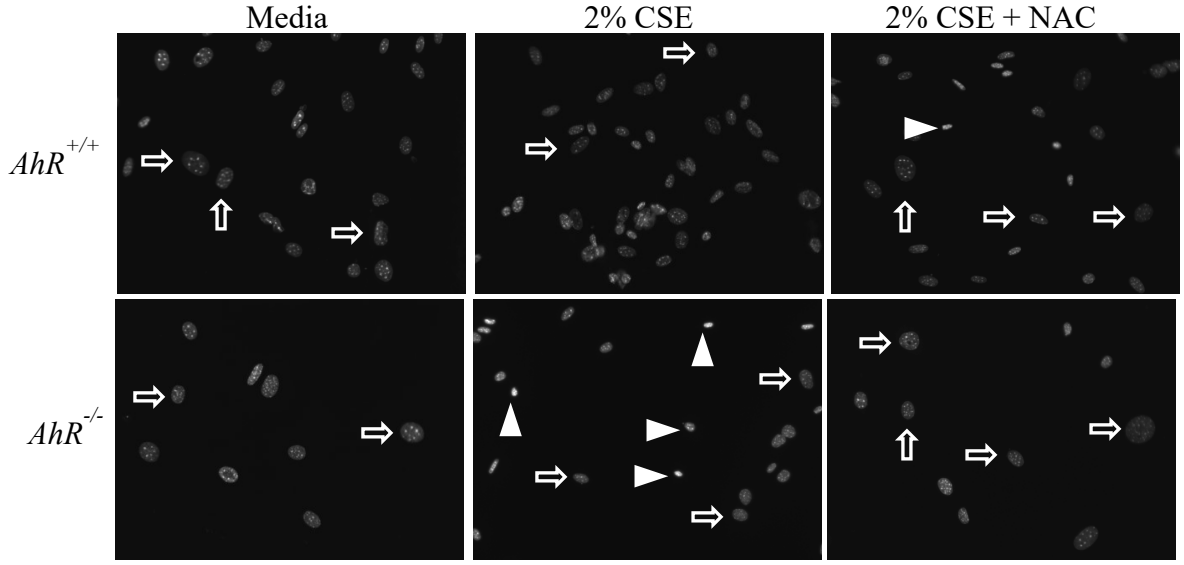


Figure 3.1. There is increased oxidative stress in AhR-deficient fibroblasts and epithelial cells exposed to CSE. (**A**) Flow cytometry/DCFDA—lung fibroblasts: Flow cytometric analysis of fibroblasts treated with 2% CSE for 4 h and stained with DCFDA demonstrate that there is an increase in fluorescence intensity, representing increasing ROS production predominantly in $AhR^{-/-}$ fibroblasts. A representative histogram from a single experiment is shown. (**B**) ROS production-lung fibroblasts: Quantification of ROS production revealed that 2% CSE significantly increased ROS production in $AhR^{-/-}$ cells compared with both untreated cells (** $p < 0.01$) and CSE-exposed $AhR^{+/+}$ fibroblasts ($$ $p < 0.01$). Results are expressed as mean ± SEM of five independent experiments. (**C**) Flow cytometry/DCFDA-A549: Flow cytometric analysis of A549 cells treated with 5 or 10% CSE for 4 h and stained with DCFDA demonstrate that there is an increase in fluorescence intensity, representing increasing ROS production more noticeably in A549-AhR^{ko} than in the AhR-expressing parent cell line. A representative histogram from a single experiment is shown. (**D**) ROS production—A549: Quantification of ROS production revealed that 10% CSE significantly increases ROS production in A549-AhRko cells compared with untreated cells (** $p < 0.01$) as well as CSE- exposed $A549^{Parent}$ cells ($ $p < 0.05$). Results are expressed as mean ± SEM of four independent experiments.

Figure 3.2

A. Chromatin Condensation- NAC



B. Quantification-NAC

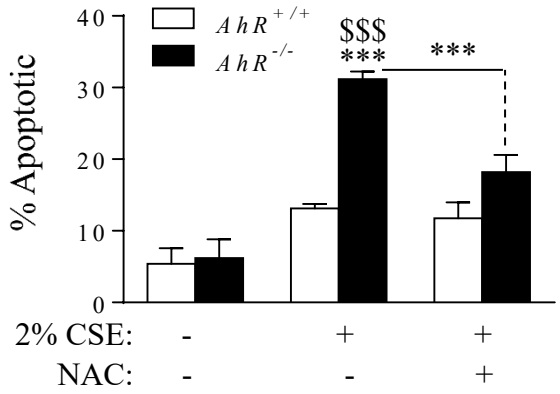


Figure 3.2. Pretreatment with the antioxidant NAC abrogates CSE-induced chromatin condensation in *AhR*$^{-/-}$ lung fibroblasts. (**A**) Chromatin condensation-NAC: In *AhR*$^{-/-}$ lung fibroblasts pretreated with NAC, there was a noticeable reduction in brightly fluorescent nuclei (condensed chromatin; arrowheads) compared with cells exposed to 2% CSE alone. Arrows indicate nuclei without chromatin condensation. (**B**) Quantification-NAC: There was a significant reduction in chromatin condensation in AhR$^{-/-}$ cells treated with NAC together with 2% CSE versus CSE alone (*** $p < 0.001$; $$$ $p < 0.001$ comparing CSE-exposed *AhR*+/+ and *AhR*$^{-/-}$ fibroblasts). Results are expressed as mean ± SEM of three experiments.

3.2 Cigarette smoke differentially regulates gene expression associated with oxidative stress and antioxidant defense between *AhR*$^{+/-}$ and *AhR*$^{-/-}$ lung fibroblasts

The data presented here, as well as in previous works (48) support that the AhR suppresses cigarette smoke-induced oxidative stress, a feature that contributes to the ability of the AhR to promote lung cell survival. As key elements of the antioxidant response (e.g. GSH levels, Nrf2 activation) were similar between *AhR*$^{+/+}$ and *AhR*$^{-/-}$ cells (48), the mechanism by which the AhR suppresses ROS production and hence cell death remained unclear. Therefore, we next addressed the possibility that there is differential regulation of gene expression associated with oxidative stress and antioxidant defenses between cigarette smoke between *AhR*$^{+/-}$ and *AhR*$^{-/-}$ cells. To evaluate this, we performed an RT^2-PCR array containing numerous transcripts related to oxidative stress, including genes related to ROS metabolism (Sod1 [CuZnSod] and Sod2 [MnSod]) and oxidant defense (Nqo1, Srxn1, Prdx6). The majority of genes examined had a less than 3-fold change in expression between CSE-exposed *AhR*$^{+/-}$ and *AhR*$^{-/-}$ cells, including glutathione Gpx1, NADPH oxidase (Nox) 1 and thioredoxin reductase (Txnrd) 3 (Table 3.1). Of the genes analyzed on the array, most notable was the robust induction of Nqo1 (≈ 39-fold) in *AhR*$^{+/+}$ fibroblasts exposed to 2% CSE for 6 hours (Fig. 3.3, white bar). There was considerably less Nqo1 mRNA induction (≈ 6.5-fold) in *AhR*$^{-/-}$ cells exposed to 2% CSE (Fig. 3.3, black bars). Also strongly induced by CSE in the *AhR*$^{+/-}$ cells relative to *AhR*$^{-/-}$ cells was Srxn1 (≈8 versus 4.9, respectively) (Fig. 3.3). There was little difference in the induction of Sod1 or Sod2 mRNA between CSE-exposed *AhR*$^{+/-}$ and *AhR*$^{-/-}$ fibroblasts. Next, we performed qRT-PCR analysis on samples from individual experiments to confirm the expression data obtained from the qPCR array. We included in our analysis Sod1, Sod2, Nqo1 and Srxn1. The choice of these genes was based on our previous data demonstrating lower SOD1 and SOD2 protein expression in *AhR*$^{-/-}$ cells (45) as well as the well-characterized (Nqo1)

versus unknown (Srxn1) regulation by the AhR. While there was little difference in Sod1 or Sod2 mRNA expression or induction by 2% CSE between *AhR*$^{+/+}$ and *AhR*$^{-/-}$ fibroblasts (Fig. 3.4A and 3.4B), there was a significant increase in Srxn1 and Nqo1 mRNA only in *AhR*$^{+/+}$ fibroblasts (Fig. 3.4C and 3.4D). *Cyp1a1* mRNA expression was used as an internal control for AhR activation by 2% CSE, which was also significantly increased only in the *AhR*$^{+/+}$ cells (Fig. 3.4F). We next compared the response of 2% CSE with that of B[*a*]P, a classic AhR ligand that induces DRE-dependent transcriptional responses, including induction of *Cyp1a1* and *Nqo1* expression (140,150). Similar to that observed with exposure to 2% CSE, there was no significant difference in Sod1 or Sod2 mRNA expression following B[*a*]P exposure between *AhR*$^{+/+}$ and *AhR*$^{-/-}$ lung fibroblasts (Fig. 3.5A and 3.5B). There was also no significant difference in Srxn1 mRNA expression between *AhR*$^{+/+}$ and *AhR*$^{-/-}$ fibroblasts exposed to B[*a*]P (Fig. 3.5C) whereas B[*a*]P significantly increased Cyp1a1 and Nqo1 expression in *AhR*$^{+/+}$ fibroblasts only (Fig. 3.5D and 3.5E). These data confirm the results from the qPCR array, which together support a differential role for the AhR in the regulation of select antioxidant genes by cigarette smoke.

Table 3.1. Gene expression changes in *AhR*$^{-/-}$ and *AhR*$^{+/-}$ lung fibroblasts exposed to 2% CSE for 6 hours. Genes strongly induced (fold regulation > 3) are not included in this table. Values represent fold-regulation compared media-exposed cells and are normalized to β-actin and GAPDH.

Gene Name	Gene Symbol	Gene Bank	FOLD REGULATION *AhR*$^{+/-}$	*AhR*$^{-/-}$
Glutathione peroxidase 8 (putative)	Gpx8	NM_027127	-1.4	-1.3
Aminoadipate-semialdehyde synthase	Aass	NM_013930	-2.6	-4
Amyotrophic lateral sclerosis 2	Als2	NM_028717	1.9	-1.7
Adenomatosis polyposis coli	Apc	NM_007462	-1.4	-2
Apolipoprotein E	Apoe	NM_009696	-1.9	-2.6
Ataxia telangiectasia and rad3 related	Atr	NM_019864	-2	-1.7
Catalase	Cat	NM_009804	3.7	2.5
Xin actin-binding repeat containing 1	Xirp1	NM_001081339	-2	-3.5
Cathepsin B	Ctsb	NM_007798	-1.5	-1.7
Cytochrome b-245, alpha polypeptide	Cyba	NM_007806	-1.9	-2.1
Cytoglobin	Cygb	NM_030206	-1.3	-1.5
Dynamin 2	Dnm2	NM_001039520	-2.5	-2.8
Dual oxidase 1	Duox1	XM_130483	-3	-1.5
EH-domain containing 2	Ehd2	NM_153068	-3.5	-2.8
Excision repair cross-complementing rodent repair deficiency, complementation group 2	Ercc2	NM_007949	-1.7	-2.6
Excision repair cross-complementing rodent repair deficiency, complementation group 6	Ercc6	NM_001081221	-1.9	-2.3
Fanconi anemia, complementation group C	Fancc	NM_007985	-1.6	-1.9
Growth factor receptor bound protein 2-associated protein 1	Gab1	NM_021356	-1.5	-1.6
Glutathione peroxidase 1	Gpx1	NM_008160	-1.9	-1.4
Glutathione peroxidase 2	Gpx2	NM_030677	-1.5	-2.5
Glutathione peroxidase 3	Gpx3	NM_008161	-1.7	-2.1
Glutathione peroxidase 5	Gpx5	NM_010343	-2	-3.5
Glutathione peroxidase 6	Gpx6	NM_145451	-2	-3.5
Glutathione peroxidase 7	Gpx7	NM_024198	-2	-3.5
Glutathione reductase	Gsr	NM_010344	2.3	-1.1

Hemoglobin, theta 1	Hbq1	NM_175000	-2	-3.5
Isocitrate dehydrogenase 1 (NADP+), soluble	Idh1	NM_010497	1.5	-1.5
Intraflagellar transport 172 homolog	Ift172	NM_026298	-2.1	-2.5
Interleukin 19	Il19	NM_001009940	-2.5	-3.5
Interleukin 22	Il22	NM_016971	-2	-3.5
Kinesin family member 9	Kif9	NM_010628	-1.7	-2.1
Lactoperoxidase	Lpo	NM_080420	-2	-3.5
Myoglobin	Mb	NM_013593	-1.1	-3
Myeloperoxidase	Mpo	NM_010824	-2	-3.5
Membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)	Mpp4	NM_145143	-1.6	-3.5
Neutrophil cytosolic factor 2	Ncf2	NM_010877	-1.9	-1.1
Neuroglobin	Ngb	NM_022414	-1.1	1.1
Nitric oxide synthase 2, inducible	Nos2	NM_010927	1	-2.5
NADPH oxidase 1	Nox1	NM_172203	-2	-2.1
NADPH oxidase 4	Nox4	NM_015760	-3	-2.1
NADPH oxidase activator 1	Noxa1	NM_172204	-2	-3.5
NADPH oxidase organizer 1	Noxo1	NM_027988	1.1	-1.5
Nudix (nucleoside diphosphate linked moiety X)-type motif 15	Nudt15	NM_172527	-2.5	-2.5
Nucleoredoxin	Nxn	NM_008750	-1.1	-2
Parkinson disease (autosomal recessive, early onset) 7	Park7	NM_020569	-2.5	-2.5
Protein phosphatase 1, regulatory (inhibitor) subunit 15b	Ppp1r15b	NM_133819	-1.7	-3
Peroxiredoxin 1	Prdx1	NM_011034	1.3	1.1
Peroxiredoxin 2	Prdx2	NM_011563	-2.5	-2.1
Peroxiredoxin 3	Prdx3	NM_007452	-1.9	-2.6
Peroxiredoxin 4	Prdx4	NM_016764	1	-1
Peroxiredoxin 5	Prdx5	NM_012021	-1.3	-1.6
Prion protein	Prnp	NM_011170	-2.8	-2.6
Proteasome (prosome, macropain) subunit, beta type 5	Psmb5	NM_011186	-1.2	-2
Prostaglandin-endoperoxide synthase 1	Ptgs1	NM_008969	-1.7	-1.9
Prostaglandin-endoperoxide synthase 2	Ptgs2	NM_011198	3.2	-1.1
Recombination activating gene 2	Rag2	NM_009020	-2	-3.5
Stearoyl-Coenzyme A desaturase 1	Scd1	NM_009127	-2.1	-1.7
Serine (or cysteine) peptidase inhibitor, clade B, member 1b	Serpinb1b	NM_173052	-1.1	-1.7

Solute carrier family 38, member 1	Slc38a1	NM_134086	1.6	-1.2
Solute carrier family 41, member 3	Scl41a3	NM_027868	-2.1	-1.7
Tropomodulin 1	Tmod1	NM_021883	-1.4	-1.5
Thyroid peroxidise	Tpo	NM_009417	-2.1	-3.5
Thioredoxin interacting protein	Txnip	NM_023719	1.1	-2.1
Thioredoxin reductase 1	Txnrd1	NM_015762	3.2	1.2
Thioredoxin reductase 2	Txnrd2	NM_013711	-1.7	-2.6
Thioredoxin reductase 3	Txnrd3	NM_153162	-1.3	-1.9
Uncoupling protein 3 (mitochondrial, proton carrier)	Ucp3	NM_009464	-2	3.5
Vimentin	Vim	NM_011701	1.1	1.1
Xeroderma pigmentosum, complementation group A	Xpa	NM_011728	1	-1.5
Zinc finger, MYND domain containing 17	Zmynd17	XM_127602	1.1	-1.2

Figure 3.3

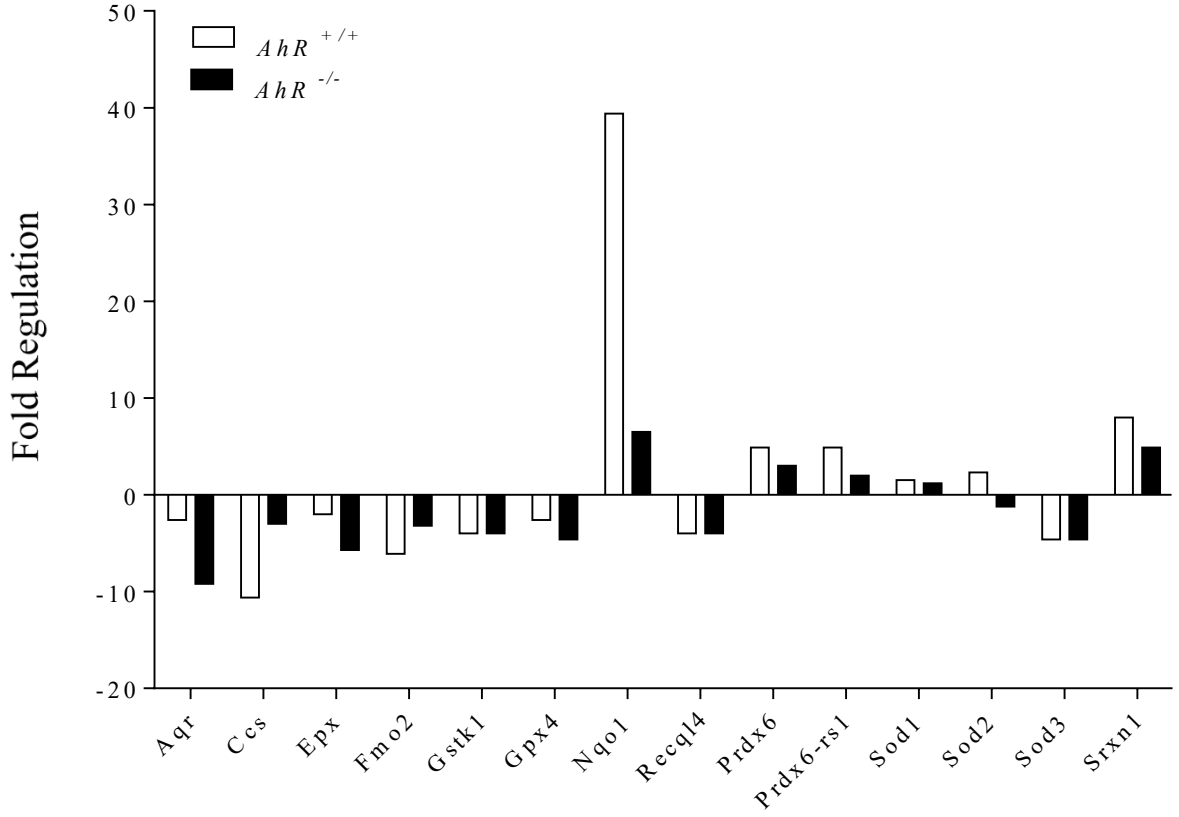


Figure 3.3. Differential expression of CSE-induced mRNA levels evaluated by RT2-PCR array. *AhR*$^{-/-}$ and *AhR*$^{+/+}$ fibroblasts were exposed to 2% CSE for 6 h as described above and cell lysates were processed for PCR analysis utilizing a commercial array containing approximately 88 genes. Representative genes were selected for graphical representation to depict differential expression between *AhR*$^{-/-}$ and *AhR*$^{+/+}$ cells. Values are presented as the fold change compared with respective media control and were normalized to GAPDH and β-actin.

Figure 3.4

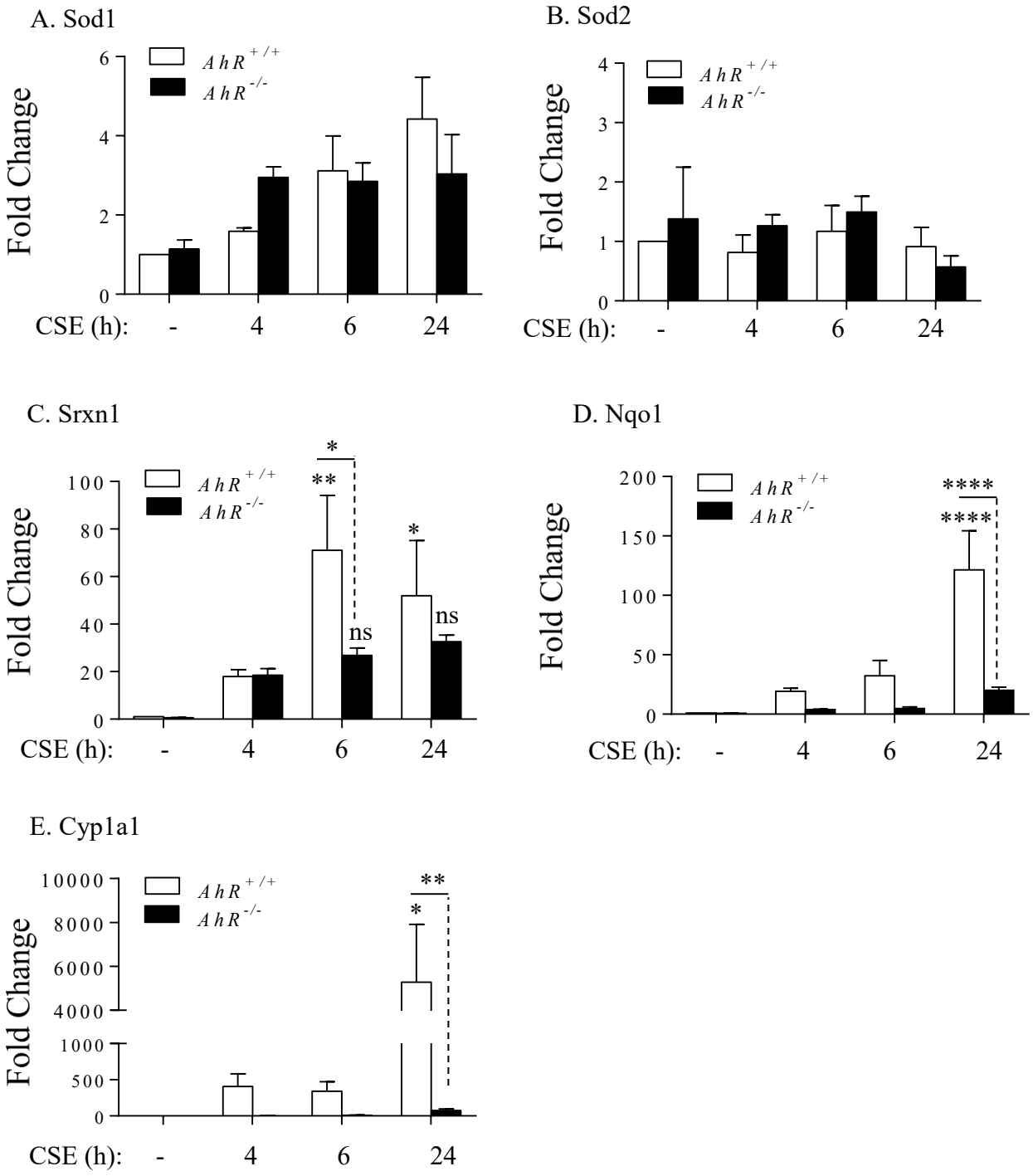

A. Sod1
B. Sod2
C. Srxn1
D. Nqo1
E. Cyp1a1
Fold Change
AhR+/+
AhR-/-
CSE (h): - 4 6 24
ns
*
**
****

Figure 3.4. CSE-induced mRNA of antioxidant genes in *AhR*$^{-/-}$ compared with *AhR*$^{+/+}$ fibroblasts. *AhR*$^{-/-}$ and *AhR*$^{+/+}$ fibroblasts were exposed to 2% CSE for 4, 6, and 24 h and gene expression was analyzed by qRT-PCR. There was no significant change in the expression of (**A**) Sod1 or (**B**) Sod2. (**C**) Srxn1: CSE significantly increased Srxn1 expression only in *AhR*$^{+/+}$ fibroblasts (** $p < 0.01$ compared with untreated; * $p < 0.05$). (**D**) Nqo1: Exposure to 2% CSE significantly increased Nqo1 levels in *AhR*$^{+/+}$ fibroblasts (**** $p < 0.0001$ compared with untreated). (E) *Cyp1a1*: 2% CSE significantly increased Cyp1a1 levels in *AhR*$^{+/+}$ fibroblasts (* $p < 0.05$ compared with untreated; ** $p < 0.01$). Results are expressed as the mean ± SEM of three independent experiments and all values were normalized to β-actin.

Figure 3.5

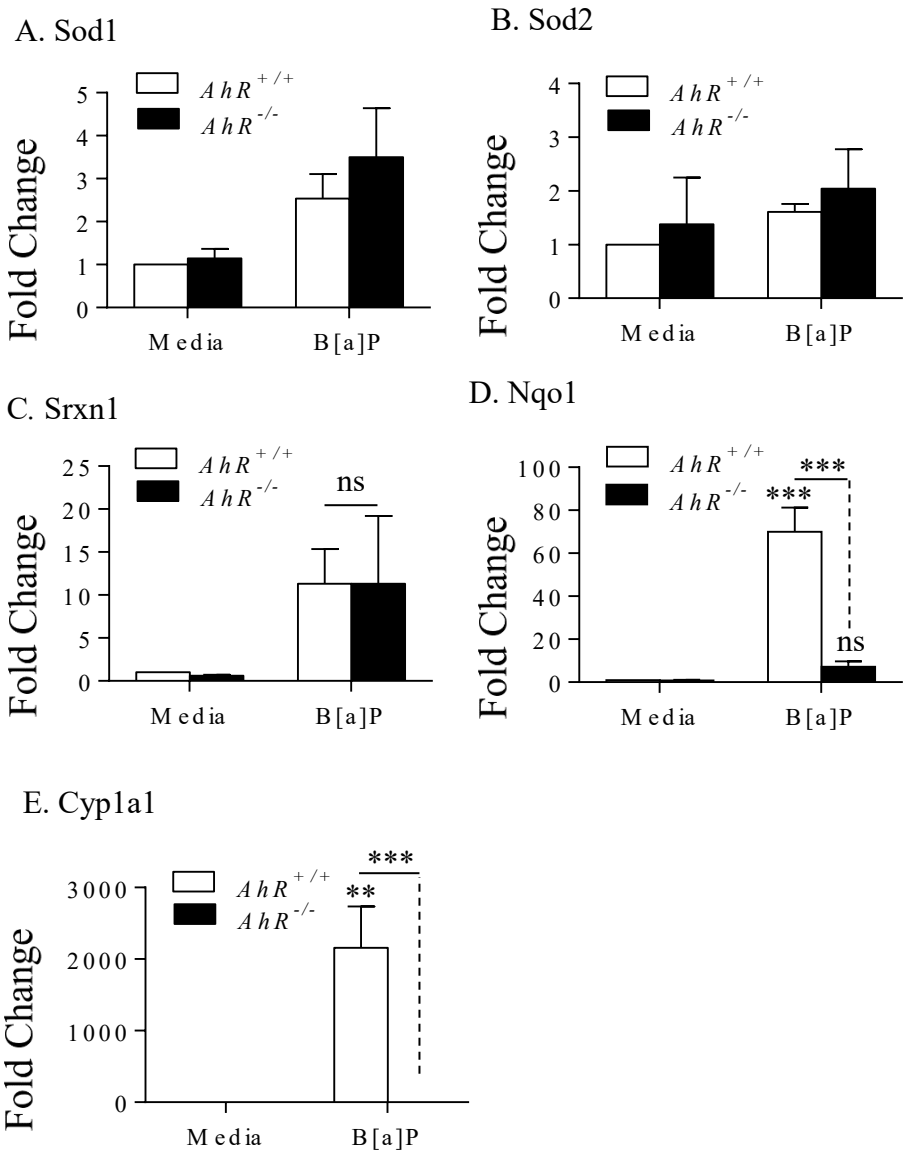


Figure 3.5. B[*a*]P exposure differentially increases expression of Cyp1a1 and Nqo1 but not Srxn1 in lung fibroblasts. *AhR*$^{-/-}$ and *AhR*$^{+/+}$ fibroblasts were exposed to 1 μM of B[*a*]P for 6 hours, after which gene expression was analyzed by qRT-PCR. There was no significant difference in (**A**) Sod1 or (**B**) Sod2 mRNA between B[*a*]P-exposed *AhR*$^{-/-}$ and *AhR*$^{+/+}$ cells. (**C**) Srxn1: There was no significant difference in Srxn1 expression between *AhR*$^{-/-}$ and *AhR*$^{+/+}$ fibroblasts. (**D**) Nqo1: B[*a*]P significantly increased Nqo1 mRNA in *AhR*$^{+/+}$ fibroblasts (*** $p < 0.001$; compared with untreated; ns = *AhR*$^{-/-}$ fibroblasts compared with untreated). (**E**) Cyp1a1: B[*a*]P significantly increased Cyp1a1 mRNA only in *AhR*$^{+/+}$ fibroblasts (** $p < 0.01$ compared with untreated; *** $p < 0.001$). Results are expressed as the mean ± SEM of three independent experiments and all values were normalized to β-actin.

3.3 AhR-dependent induction of Srxn1 expression and attenuation of oxidative stress by CSE is independent of the DRE

We have published that the AhR exerts some of its protective abilities against cigarette smoke independent of DRE binding despite the fact that both cigarette smoke and classic ligands including B[*a*]P activate AhR-dependent transcription (e.g. Cyp1a1) (140,145). Our data showing that the differential induction of Srxn1 expression by CSE, but not B[*a*]P, requires AhR expression (Figs. 3.4-3.5) suggests that non-DRE dependent mechanisms may contribute to the regulation of Srxn1 and subsequent attenuation of oxidative stress by the AhR. To first determine whether the induction of Srxn1 by CSE is DRE-independent, we utilized primary lung fibroblasts derived from $AhR^{DBD/DBD}$ mice, which express an AhR that is incapable of binding the DRE, and analyzed gene expression profiles after exposure to 2% CSE. Similar to $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts, there was no significant difference in Sod1 or Sod2 mRNA between control ($AhR^{DBD/B6}$) and $AhR^{DBD/DBD}$ cells (Fig. 3.6A and 3.6B). While there was a significant increase in Srxn1 expression in both $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ fibroblasts after exposure to CSE for 6 hours, there was no significant difference between them (Fig. 3.6C). Consistent with well-established regulation due to DRE-dependent transcription, there was a significant and robust induction of Nqo1 mRNA only in $AhR^{DBD/B6}$ fibroblasts exposed to CSE (Fig. 3.6D). Thus, we show for the first time that the expression of Srxn1 mRNA by cigarette smoke in lung fibroblasts is controlled through an AhR-dependent, but DRE-independent, mechanism. We next analyzed whether the heightened CSE-induced oxidative stress due to AhR deficiency (Fig. 3.1) is also DRE-dependent. $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ fibroblasts were exposed to 2% CSE for 4 hours and ROS production analyzed by flow cytometry/fluorescence of the ROS-sensitive dye H_2DCFDA. Despite the slight induction in ROS in both $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ cells after exposure to CSE, there was also no significant difference

in ROS levels between the $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ fibroblasts (Fig. 3.7). This indicates that the suppression of oxidative stress in response to cigarette smoke requires AhR expression but it independent of the DRE.

Figure 3.6

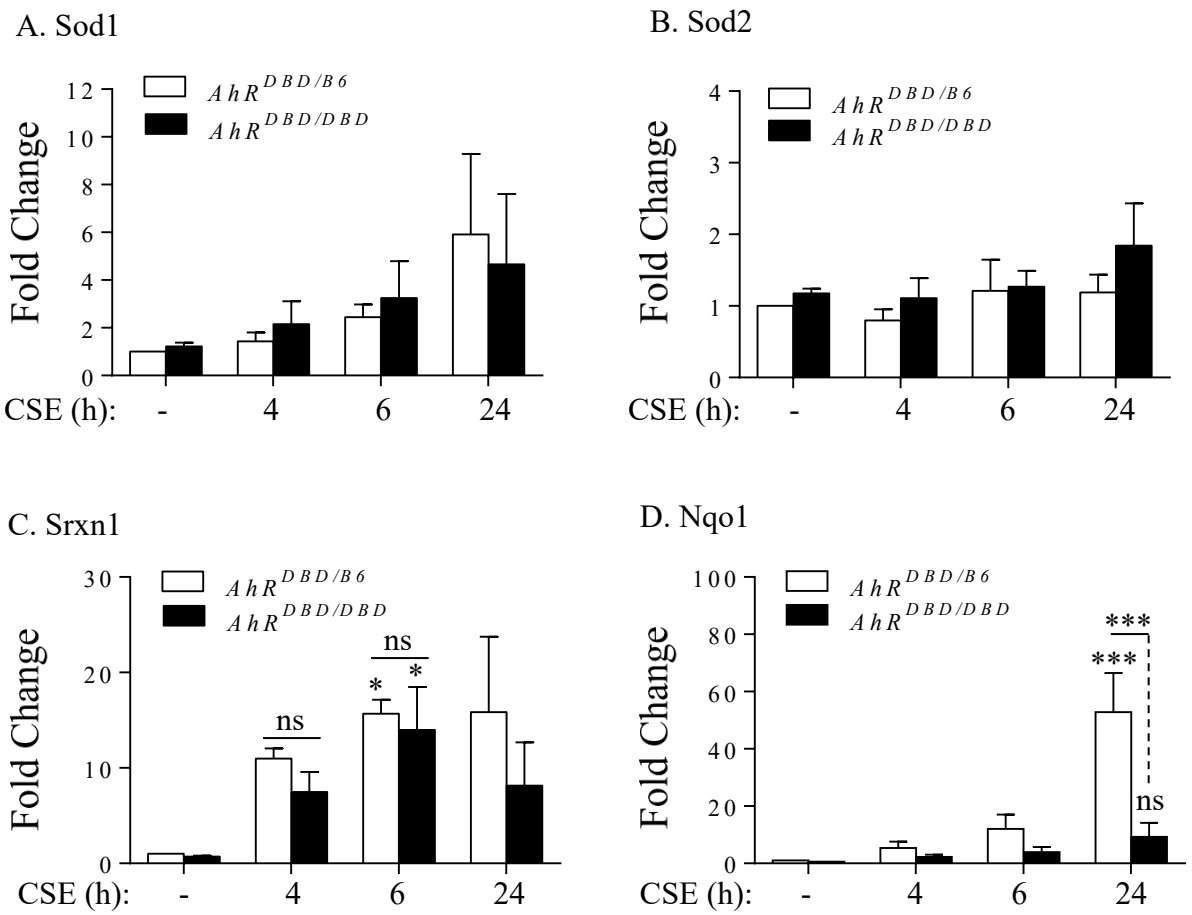


Figure 3.6. CSE-induced mRNA expression in *AhR*$^{DBD/DBD}$ versus *AhR*$^{DBD/B6}$ fibroblasts. *AhR*$^{DBD/B6}$ and *AhR*$^{DBD/DBD}$ fibroblasts were exposed to 2% CSE and mRNA expression for select genes analyzed by qRT-PCR as described in Materials and methods. Expression of (**A**) Sod1 and (**B**) Sod2 was not significantly different between *AhR*$^{DBD/DBD}$ and *AhR*$^{DBD/B6}$ fibroblasts. (**C**) Srxn1: There was a significant increase in Srxn1 mRNA expression in both *AhR*$^{DBD/B6}$ and *AhR*$^{DBD/DBD}$ fibroblasts exposed to 2% CSE for 6 h (* $p < 0.05$ compared with respective control). This induction was not different between the CSE-exposed *AhR*$^{DBD/B6}$ and *AhR*$^{DBD/DBD}$ cells (ns) at any time-point. (**D**) Nqo1: There was a significant increase in Nqo1 mRNA in response to 2% CSE only in the *AhR*$^{DBD/B6}$ fibroblasts (*** $p < 0.001$ compared with media only). No significant induction of Nqo1 mRNA in *AhR*$^{DBD/DBD}$ cells was found in any time-point (ns). Results are expressed as the mean ± SEM of three independent experiments and all values were normalized to β-actin.

Figure 3.7

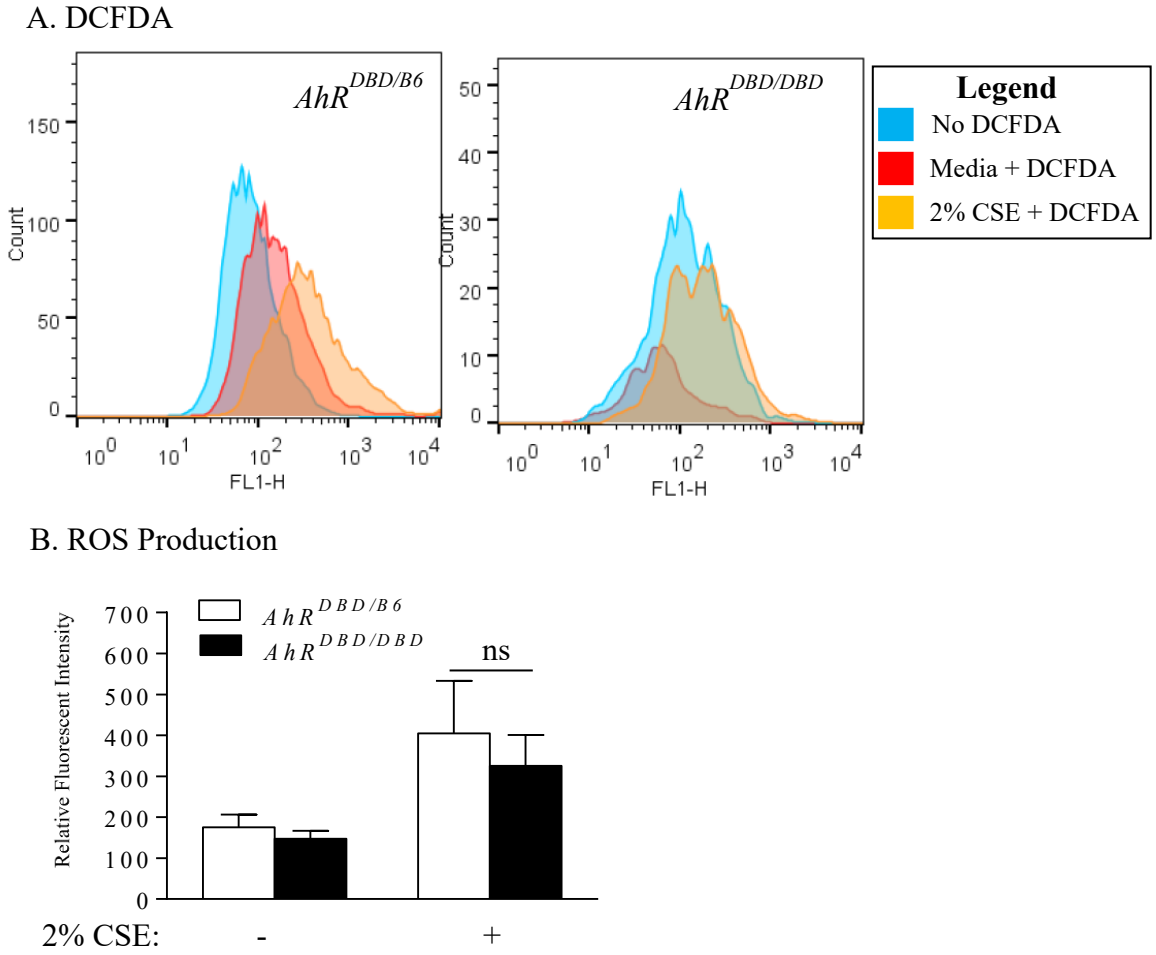


Figure 3.7. Suppression of CSE-induced alteration in ROS production is independent of the DRE. *AhR*$^{DBD/B6}$ and *AhR*$^{DBD/DBD}$ fibroblasts were exposed to 2% CSE for 4 hours and flow cytometric analysis with H_2DCFDA (DCFDA) utilized as an indicator of cellular oxidative stress. (**A**) DCFDA: There was little apparent difference in the fluorescence intensity (FL1) between CSE-exposed *AhR*$^{DBD/B6}$ and *AhR*$^{DBD/DBD}$ lung fibroblasts. Representative histograms are shown. (**B**) ROS production: There was no significant difference in ROS production evoked by exposure to 2% CSE between the *AhR*$^{DBD/B6}$ and *AhR*$^{DBD/DBD}$ cells. Results are expressed as the mean ± SEM of three independent experiments.

3.4 AhR regulation of Srxn1 induction by CSE is not directly mediated by Nrf2

Numerous studies indicate cross-talk between the AhR and Nrf2 (141–143). Both are activated by cigarette smoke and activate shared gene batteries governing antioxidant and Phase II detoxification pathways. Nrf2 is a known activator of Srxn1 in the lung (66). Our prior understanding of the relationship between the AhR-Nrf2 antioxidant defense pathway becomes uncertain when taken together with previous results from our lab. It has been shown that between $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts treated with CSE, that there is no difference between Nrf2 protein expression, nor differences in Nrf2 translocation to the nucleus (48). We further investigated whether AhR regulation of Srxn1 was due to control of Nrf2 function in order to clarify the relationship between the AhR and Nrf2.

The first steps were to corroborate findings with our previous work on Nrf2 expression between $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts treated with CSE. There was an increase (≈ 4-6-fold) in mRNA expression of Nrf2 in response to 2% CSE, but there was little difference in expression between $AhR^{+/-}$ and $AhR^{-/-}$ *f*ibroblasts or $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ fibroblasts (Fig. 3.8A and B). We next evaluated whether there was reciprocal regulation of the AhR/Nrf2 pathways and if this regulation was different between AhR-expressing and AhR-deficient cells. Using a BEAS-2B cell line stably transfected with a Nrf2-luciferase construct, we established that both the classical Nrf2 agonist SFN and CSE significantly increase Nrf2 activity (Fig. 3.8C). SFN exhibited no AhR agonist activity in lung fibroblasts, with little change in Cyp1a1 mRNA expression in $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts (Fig. 3.8D). There was, however, a robust increase in Srxn1 levels in $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts exposed to SFN (Fig. 3.8E); this increase in Srxn1 was not different between $AhR^{+/+}$ and $AhR^{-/-}$ cells. Similar to our prior results in lung fibroblasts (48), there was little

difference in both Nrf2 nuclear translocation and Srxn1 mRNA induction in response to CSE between A549^{Parent} and A549-AhR^{ko} lung epithelial cells (Fig. 3.8F and G). We used siRNA against Nrf2 in *AhR*$^{+/+}$ lung fibroblasts and evaluated Srxn1 expression (Figs. 3.9A and B). There was significantly lower induction of Srxn1 in response to SFN or CSE in siNrf2 cells than in control fibroblasts (Figs 3.9C and D). These findings reaffirm the regulatory role Nrf2 has over Srxn1 gene expression in lung cells. Nonetheless, our findings suggest a novel role for the AhR in regulation of Srxn1 in response to CS in a DRE and Nrf2 independent manner.

Figure 3.8

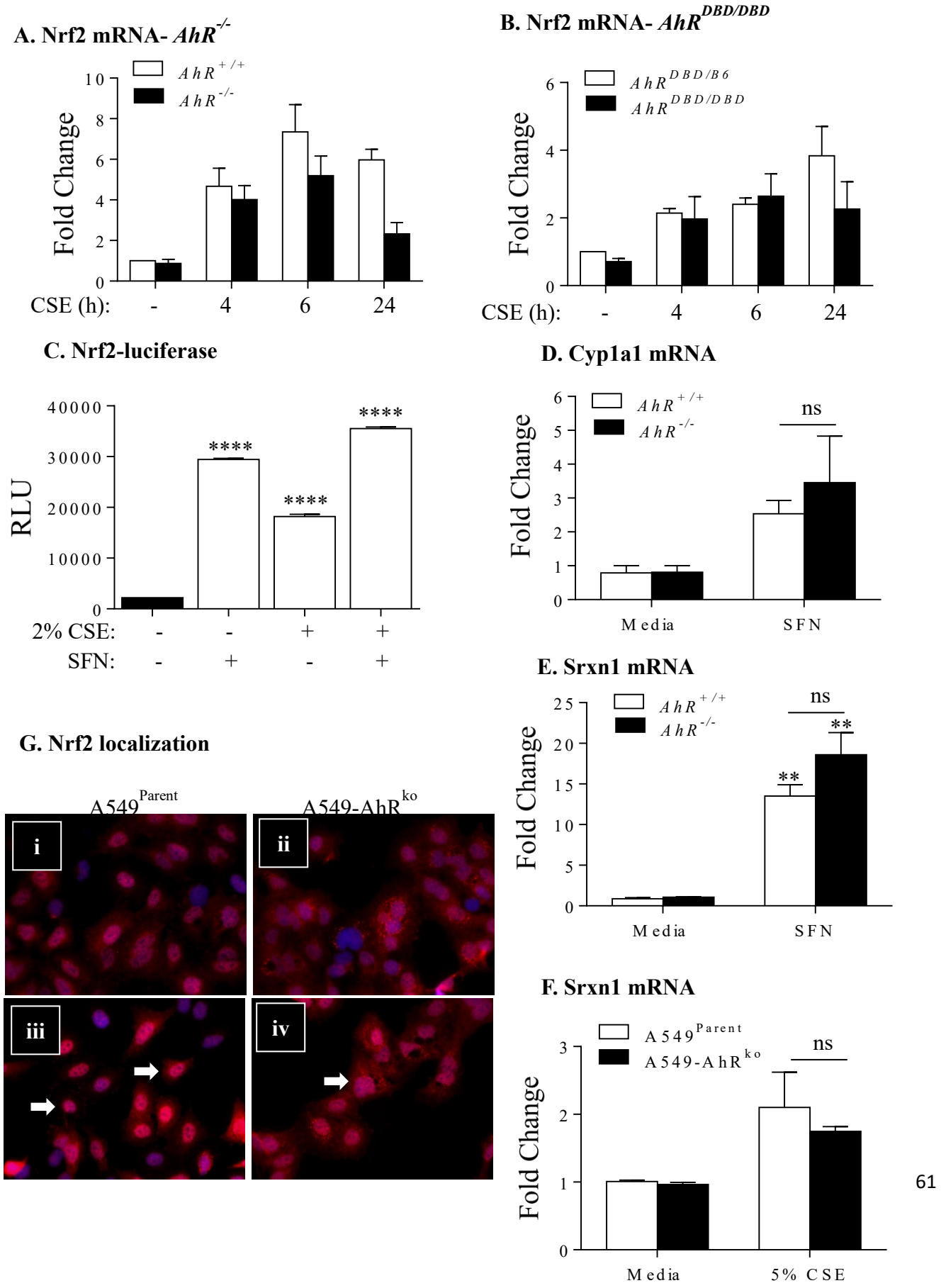

A. Nrf2 mRNA- AhR-/-
AhR+/+
AhR-/-
Fold Change
CSE (h): - 4 6 24
B. Nrf2 mRNA- AhRDBD/DBD
AhRDBD/B6
AhRDBD/DBD
Fold Change
CSE (h): - 4 6 24
C. Nrf2-luciferase
RLU
2% CSE: - - + +
SFN: - + - +
D. Cyp1a1 mRNA
AhR+/+
AhR-/-
ns
Fold Change
Media
SFN
E. Srxn1 mRNA
AhR+/+
AhR-/-
ns
Fold Change
Media
SFN
F. Srxn1 mRNA
A549Parent
A549-AhRko
ns
Fold Change
Media
5% CSE
G. Nrf2 localization
A549Parent
A549-AhRko
i
ii
iii
iv

Figure 3.8. AhR-independent regulation of Nrf2. (**A**) Nrf2 mRNA- *AhR*$^{-/-}$: There was an increase in Nrf2 mRNA in lung fibroblasts in response to 2% CSE (≈ sixfold), with little difference between *AhR*$^{-/-}$ and *AhR*$^{+/-}$ cells. (**B**) Nrf2 mRNA- *AhR*$^{DBD/DBD}$: There was induction in Nrf2 mRNA in response to CSE in fibroblasts derived from *AhR*$^{DBD/DBD}$ and control mice but little difference between the cells. (**C**) Nrf2-luciferase: There was a significant induction of luciferase (relative light units [RLU]) in BEAS-2B cells exposed to SFN or 2% CSE as well as a combination of SFN and CSE (**** $p < 0.0001$ compared to untreated control). (**D**) Cyp1a1 mRNA: There was little induction of Cyp1a1 mRNA in *AhR*$^{-/-}$ and *AhR*$^{+/-}$ cells exposed to SFN; ns=not significant. (**E**) Srxn1 mRNA: There was an increase in Srxn1 in response to SFN in both *AhR*$^{-/-}$ and *AhR*$^{+/-}$ cells. There was no significant difference in the relative induction of Srxn1 between *AhR*$^{-/-}$ and *AhR*$^{+/-}$ lung fibroblasts (ns). (**F**) Srxn1 mRNA: There was also no significant difference in Srxn1 induction between A549-AhR^{ko} and the AhR- expressing parent cell line (A549^{Parent}). (**G**) Nrf2 localization: In media-only cells, Nrf2 was largely cytoplasmic in both A549^{Parent} and A549-AhR^{ko} cells (panels i and ii, respectively), although nuclear staining was evident. In response to 5% CSE for 4 h, there was noticeable translocation to the nucleus (arrows) in A549^{Parent} (panel iii) and A549-AhR^{ko} cells (panel iv). Representative images are shown.

Figure 3.9

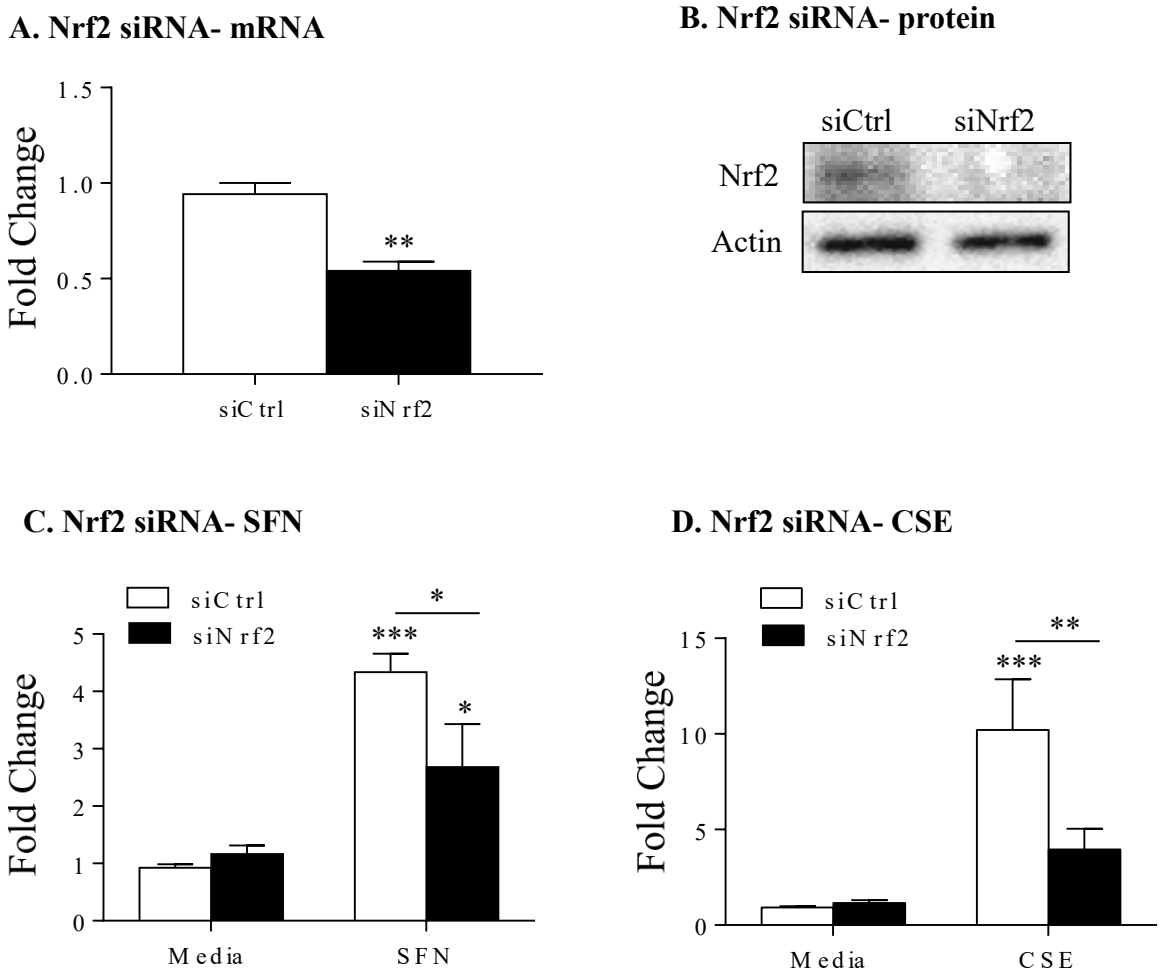


Figure 3.9. Reduction in Nrf2 levels via siRNA attenuates induction of Srxn1 in primary lung fibroblasts. (**A**) Nrf2 siRNA-mRNA: There was a significant reduction in Nrf2 mRNA after transfection with siRNA against Nrf2 (siNrf2) compared with control siRNA (siCtrl). (**B**) Nrf2 siRNA-protein: There was a noticeable decrease in Nrf2 protein levels after siNrf2 compared to scrambled siRNA (siCtrl). (**C**) Nrf2 siRNA-SFN: There was a significant induction in Srxn1 mRNA after exposure of lung fibroblasts to SFN (* $p < 0.05$; *** $p < 0.001$ compared with untreated). This induction was significantly less in siNrf2 lung fibroblasts (* $p < 0.05$ compared between SFN-treated groups). (**D**) Nrf2 siRNA-CSE: There was a signification induction in Srxn1 in response to CSE in siCtrl cells (*** $p < 0.001$); the induction of Srxn1 was significantly reduced in siNrf2 cells compared with siCtrl fibroblasts (** $p < 0.01$).

3.5 COPD derived fibroblasts have reduced AhR expression and antioxidant activity

Srxn1 and Nqo1 expression levels are reduced in lung tissue from COPD subjects compared to smokers without COPD (66,151),a feature that may increase oxidative stress in lung tissues. The expression of the AhR in COPD is not known, but given our data on the regulation of Srxn1 by the AhR, we speculate that reduced AhR expression in COPD lung fibroblasts may reduce the induction of Srxn1 by CSE exposure. Utilizing primary lung fibroblasts from never-smokers (Normal; 0 pack-years), Smokers without COPD (At Risk; 36.5±1.2 pack-years), and COPD (37.6±1.3 pack-years) subjects, we evaluated AhR protein expression. AhR was expressed in all lung fibroblasts derived from never-smokers (Fig. 10A). Expression was more variable in the smoker-derived lung fibroblasts but was readily detectable in most lung fibroblasts analyzed. In contrast, there was a noticeable reduction in the relative expression of the AhR in COPD-derived lung fibroblasts (Fig. 10A). Quantification by densitometry revealed that there was significantly less AhR protein in COPD lung fibroblasts than in either Normal or At Risk (Fig. 10B) whereas no significant difference in AhR expression between Normal and At Risk fibroblasts was found. We examined whether this decrease in AhR expression corresponded to a decrease in AhR activation by evaluating Cyp1b1 mRNA induction by CSE (140). There was a significant induction in Cyp1b1 mRNA at 3 h in the Normal and At Risk lung fibroblasts after CSE exposure (Fig. 10C). However, there was no significant induction in Cyp1b1 mRNA in the COPD lung fibroblasts at any time point examined (Fig. 10C, black bars). The AhR antagonist CH-223191 inhibited CSE-induced Cyp1b1 mRNA induction in Normal and At Risk lung fibroblasts but had minimal effect on Cyp1b1 levels in COPD cells (Fig. 10D). This confirms AhR activation as the key factor in CSE-induced Cyp1b1 expression. Additionally, there was significantly less induction of Nqo1 (Fig. 11A) and Srxn1 (Fig.

11B) mRNA in COPD lung fibroblasts in response to CSE. Taken together, our findings suggest a novel protective role for the AhR as a cellular sensor in the defense against cigarette smoke-induced oxidative stress and COPD pathogenesis.

Figure 3.10

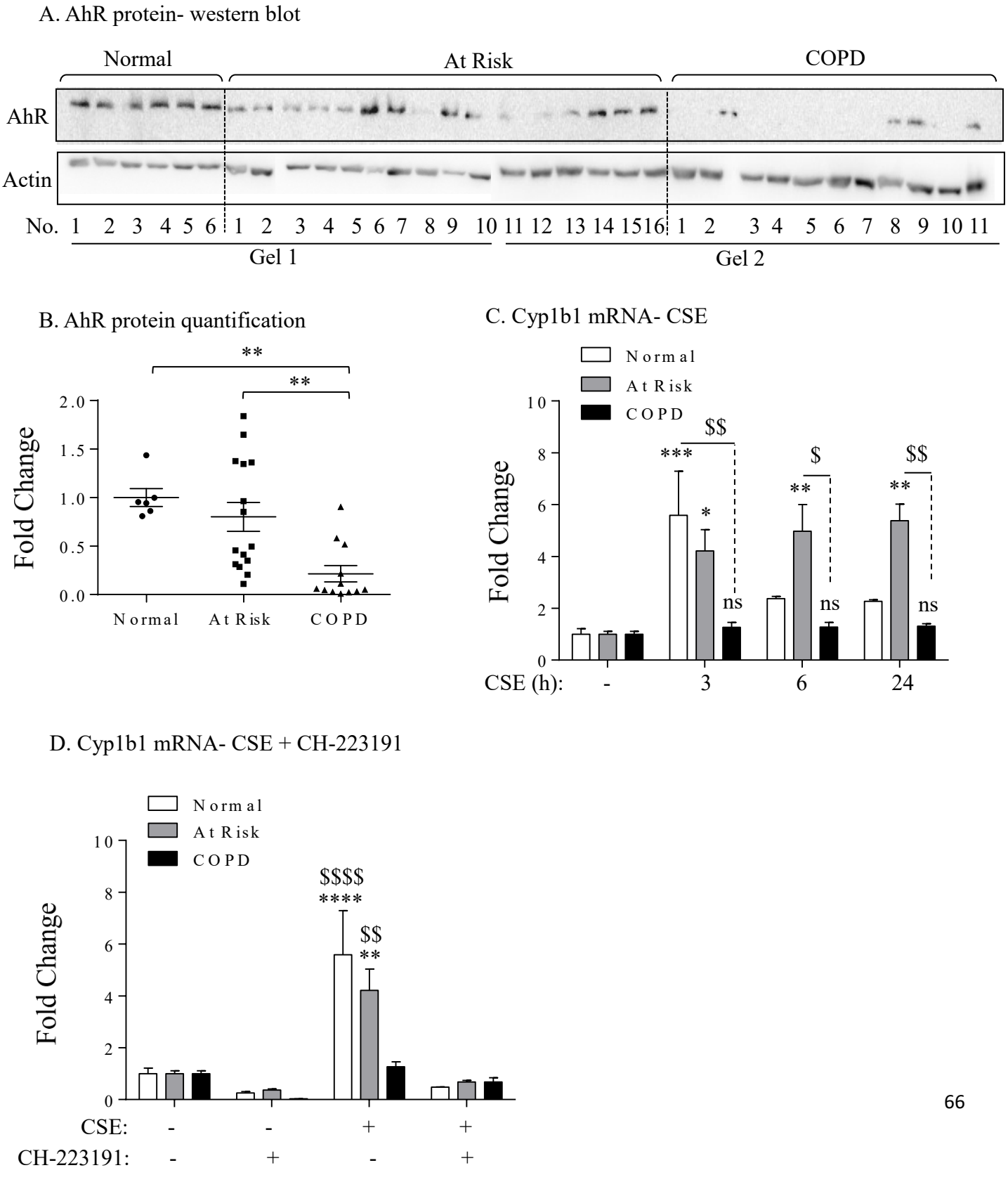

A. AhR protein- western blot
Normal
At Risk
COPD
AhR
Actin
No. 1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10 11 12 13 14 1516 1 2 3 4 5 6 7 8 9 10 11
Gel 1
Gel 2
B. AhR protein quantification
Fold Change
2.0
1.5
1.0
0.5
0.0
**
**
Normal
At Risk
COPD
C. Cyp1b1 mRNA- CSE
Normal
At Risk
COPD
Fold Change
10
8
6
4
2
0
***
*
ns
$$
**
ns
$
**
ns
$$
CSE (h): - 3 6 24
D. Cyp1b1 mRNA- CSE + CH-223191
Normal
At Risk
COPD
Fold Change
10
8
6
4
2
0
$$$$
****
$$
**
CSE: - - + +
CH-223191: - + - +

Figure 3.10. AhR protein expression is reduced in COPD-derived lung fibroblasts. (**A**) AhR protein—western blot: Fibroblasts were derived from never-smokers (Normal), smokers (At Risk), and COPD subjects and made quiescent in serum-free media prior to collection for western blot analysis. There was a relatively consistent expression in AhR protein in Normal lung fibroblasts; there was slightly less expression in At Risk fibroblasts. AhR expression was noticeably decreased in COPD-derived lung fibroblasts. No. refers to the number of fibroblasts derived from individual subjects; samples were run on two gels (Gel 1 and Gel 2). (**B**) AhR protein quantification: Values established as fold change in AhR protein normalized to actin protein expression. Densitometric analysis revealed that there was significantly less AhR protein in the COPD lung fibroblasts than in either the Normal or At Risk groups (** $p < 0.01$); there was no significant difference between the Normal and at Risk fibroblasts. Results are expressed as the mean ± SEM and each symbol represents fibroblasts from a different individual. (**C**) Cyp1b1 mRNA- CSE: Exposure of lung fibroblasts to CSE significantly increased Cyp1b1 mRNA expression in the Normal and At Risk lung fibroblasts compared with their respective controls (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); expression declined in the Normal lung fibroblasts by 6 h. There was no significant increase in Cyp1b1 mRNA in the COPD lung fibroblasts (ns) and Cyp1b1 mRNA was significantly lower compared to either Normal or At Risk fibroblasts ($ $p < 0.05$; $$ $p < 0.01$). Results are expressed as the mean ± SEM of at least three individual experiments. (**D**) Cyp1b1 mRNA-CSE + CH-223191: The significant increase in CSE-induced Cyp1b1 mRNA was prevented with CH-223191 in Normal and At Risk lung fibroblasts (**** $p < 0.0001$ and ** $p < 0.01$ compared with respective untreated; $$$$ $p < 0.0001$ or $$ $p < 0.01$ compared with COPD fibroblasts exposed to CSE + CH-223191). Results are expressed as the mean ± SEM of at least three individual experiments.

Figure 3.11

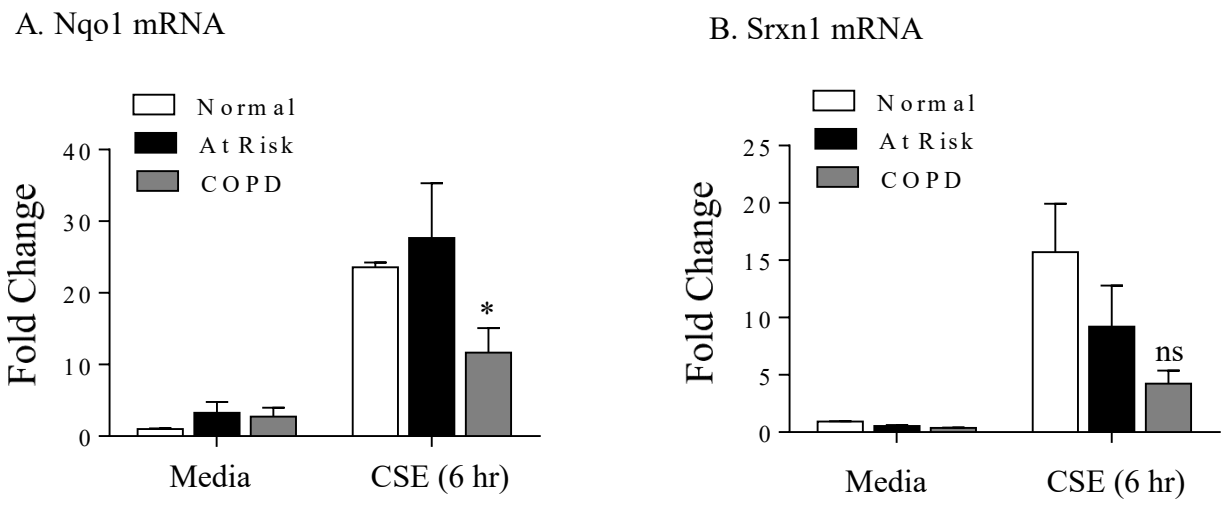


Figure 3.11. Induction of Nqo1 and Srxn1 trends lower in COPD-derived lung fibroblasts exposed to cigarette smoke. (**A**) Nqo1 mRNA: There was significantly less induction in Nqo1 mRNA in COPD-derived fibroblasts exposed to CSE than in Normal and At Risk cells (* $p < 0.05$). (**B**) Srxn1 mRNA: There was no significant increase in Srxn1 in COPD lung fibroblasts exposed to 2% CSE (ns = compared to untreated COPD fibroblasts). Results are expressed as the mean ± SEM of two or three individual experiments.

4. Discussion

For decades, the AhR has been described as the mediator of the toxic effects of such environmental contaminants as dioxins and PAH (121,152). Emerging research, however, has illustrated the necessary endogenous function of the AhR in the regulation of liver and immune development, inflammatory response, and cytoprotection (48,122,140,145,153,154). These functions can be regulated by the AhR in the absence of exogenous ligand, challenging the notion of this protein solely as the "dioxin receptor." The AhR is now understood to interact with many cellular factors, including direct binding to transcription factors such as the NF-κB member RelB (137,155), the ER (126), and Nrf2 (141,156). The AhR also has roles in regulating post-transcriptional modification through both miRNA and RNA binding protein function. This present work contributes to our understanding of the endogenous activities of the AhR by promoting a homeostatic and cytoprotective role against cigarette smoke-induced oxidative stress and apoptosis by regulation of the antioxidant genes Srxn1 and Nqo1.

Apoptosis is a key component of emphysema in COPD (22,37,157). Heightened markers for oxidative stress and apoptosis have been shown to be present in the lungs of emphysema patients. Much of the damage observed in COPD is attributed to ROS produced by cigarette smoke (12,22) and it is believed that an insufficient balance of antioxidant defense is a causal factor in COPD pathogenesis. Although 80-90% of COPD is caused by tobacco smoke inhalation, only 15-20% of tobacco smokers develop COPD, which may indicate that genetic predisposition to the pathology is a key factor (15,158). Nrf2 has been studied as a drug target for COPD treatment (73,74,82,159), and Srxn1 and Nqo1, genes prototypically thought to be governed by Nrf2, have been shown to

have lower expression in COPD patients (66,151). Additionally, antioxidants such as NAC have been proposed as a treatment option for COPD exacerbations (11,59). In addition to acting as a free radical scavenger, NAC serves to increase GSH biosynthesis by supplying cells with L-cysteine. In our own experiments, NAC was found to abrogate CSE-induced chromatin condensation (Fig 3.2). Because mouse lung fibroblasts were co-treated with NAC and CSE in this protocol, it is probable that NAC served to scavenge free radicals, preventing oxidative stress and chromatin condensation. A limitation of our study is that we did not investigate ROS levels in cells treated with CSE and NAC together, an important control. Nonetheless, prior studies indicate that there was no difference in GSH levels between $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts in response CSE exposure (48). These findings taken together suggest that oxidative stress is the cause of CSE-induced chromatin condensation and apoptosis in our study. Importantly, we have shown that $AhR^{-/-}$ mouse lung fibroblasts in addition to A549-AhR^{ko} epithelial cells have significantly heightened levels of oxidative stress following CSE exposure compared to their respective controls (Fig 3.1).

Our results show for the first time that the AhR regulates the expression of Srxn1, which may act as a key protective factor against cigarette smoke-induced oxidative stress. Srxn1 is an ATP-dependent antioxidant enzyme that functions to reactivate hyperoxidized Prx, a family of broad-spectrum peroxide-reducing enzymes. Srxn1 was also identified as capable of reducing the post-translational oxidative modification of glutathionylation of proteins, a feature that may be involved with certain degenerative chronic diseases (65). Srxn1 expression is regulated by the transcription factors AP-1, NF-κB, and Nrf2 in response to cigarette smoke, diesel exhaust particles, and both hyperoxia and hypoxia (160). Srxn1 protein expression levels were found to be significantly lower in the lungs of advanced COPD patients. Our findings establish for the first time that Srxn1 mRNA is significantly reduced in $AhR^{-/-}$ fibroblasts compared to wild-type following

CSE exposure. Interestingly, however, the classical AhR ligand B[*a*]P had no effect on the transcription of Srxn1 between $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts. In $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ fibroblasts, there was no change in Srxn1 expression following CSE exposure. Taken together, these facts suggest that a functional AhR is necessary to ensure the proper expression of Srxn1 in mouse lung fibroblasts, but this does not occur through a canonical genomic pathway. Indeed, prior to the findings of this work, Srxn1 was understood to be regulated only by the so-called "master regulator of antioxidant response," Nrf2 (66,70).

The crosstalk between Nrf2 and AhR has been studied as an important axis in the regulation of antioxidant defense. It has been shown that there is a functional DRE in the promoter region of Nrf2 (161). Likewise, there is an antioxidant response element (ARE) in the promoter for AhR (162). Under conditions of xenobiotic or oxidative stress, AhR and Nrf2 can therefore cooperate in xenobiotic metabolism, transcribing for each other as well as for such prototypical phase I and II genes as Cyp1a1 and GST, respectively. Additionally, AhR and Nrf2 have been shown to physically interact with each other, facilitating the expression of Nqo1 (156). It is suggested that both Nrf2 and the AhR expression are prerequisite for Nqo1 transcription (142). Nonetheless, our research indicates that Nqo1 mRNA levels are significantly altered in *AhR*-deficient mouse lung fibroblasts in response to CSE. B[*a*]P exposure strongly induced Nqo1 mRNA as well, and there was a significant difference between Nqo1 expression in $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ fibroblasts treated with CSE at 24 hours. This indicates that Nqo1 expression is controlled by the AhR in a genomic and classical model, in contrast to our findings with Srxn1, which did not require AhR-DNA binding or activation by a classical ligand (e.g., B[*a*]P) while still needing the presence of the AhR. In either case, Nrf2 expression and activity appear to be unnecessary.

4.1. AhR-Nrf2 Cross-Interactions and Potential Mechanisms of Srxn1 Regulation

Previous results from our lab and the present work run counter to the conventional understanding of the AhR-Nrf2 antioxidant defense pathway. We have shown that between $AhR^{+/+}$ and $AhR^{-/-}$ fibroblasts treated with CSE, there is no difference in Nrf2 protein expression or translocation to the nucleus (48). In order to further characterize the relationship between the AhR and Nrf2 in our model systems, we performed additional investigations (see **Chapter 3.4**). Based on these results, it appears that although Nrf2 is expressed and becomes activated in response to SFN or CSE treatment, there is no difference between $AhR^{+/+}$ and $AhR^{-/-}$ or $AhR^{DBD/B6}$ and $AhR^{DBD/DBD}$ fibroblasts. $A549^{Parent}$ and $A549\text{-}AhR^{ko}$ human lung epithelial cells were also used to characterize the relationship between the AhR and Nrf2 transcriptional activity. It is noteworthy that A549 cells have a homozygous mutation in Keap1, the cytosolic repressor of Nrf2 (163). In effect, A549 cells have a form of constitutive Nrf2 activity, yet despite this property, we found that $A549\text{-}AhR^{ko}$ cells displayed significantly increased oxidative stress compared to wild-type. siRNA directed against Nrf2 attenuated Srxn1 expression following SFN treatment, reaffirming the established regulatory role Nrf2 has over Srxn1. Our finding that Srxn1 expression was significantly lowered in AhR-deficient cells, regardless of Nrf2 expression and activation, underscores the putative importance of AhR activity in Srxn1 gene regulation. These findings parallel others' work investigating the cross-interaction between Nrf2 and the AhR, whereby both may share importance in the regulation of Nqo1 expression (142).

Key questions remain as to how Srxn1 is regulated by the AhR. Srxn1, which was previously understood to be regulated in a Nrf2-ARE DNA-binding dependent manner, was found to be significantly lowered in AhR-deficient mouse lung fibroblasts exposed to CSE. The classical

AhR activator B[*a*]P did not have an effect on Srxn1 mRNA expression. Similarly, in fibroblasts from mice with an AhR unable to bind to DNA and activated in a classical manner ($AhR^{DBD/DBD}$), Srxn1 was induced by CSE, but without any change between $AhR^{DBD/B6}$ controls and $AhR^{DBD/DBD}$. Interestingly, there was no difference in oxidative stress levels between $AhR^{DBD/B6}$ controls and $AhR^{DBD/DBD}$ fibroblasts. These findings indicate that although the AhR is necessary for Srxn1 expression, this may occur in a noncanonical or nongenomic manner. The nongenomic effects of the AhR, for example, extend to the regulation of microRNA-196a, leading to protection against CSE-induced apoptosis (139). It is conceivable that the regulation of certain miRNA by the AhR might lead to alterations in expression of Srxn1. Bioinformatics applications such as targetscan.org predict that miR-146a may interact with murine Srxn1 mRNA. miR-146a has been implicated in protection against CSE-induced inflammation in mouse lung fibroblasts (164). It is plausible that differential regulation of Srxn1 mRNA in CSE-treated $AhR^{-/-}$ fibroblasts may be governed by changes in miR-146a expression. Our lab had also shown that the AhR regulates miR-96, another miRNA predicted to target Srxn1 (165). $AhR^{-/-}$ mice exposed to CS had significantly heightened levels of pulmonary miR-96, while expressing less Srxn1 mRNA compared to $AhR^{+/-}$ controls. The present study has only correlated Srxn1 expression to miR-96; further exploration could reveal a direct role for miR-96 in the regulation of Srxn1 by the AhR.

AhR-mediated post-transcriptional regulation of Srxn1 expression may also come from changes in RNA binding protein activity. RNA binding proteins, which bind to AU-rich elements in the 3'UTR of mRNA transcripts, stabilize transcripts for protein translation. The nuclear translocation of HuR was shown by our lab to be controlled by the AhR (140). AhR-deficient cells had increased cytoplasmic HuR in response to CSE, resulting in increased translation of inflammatory mRNA. HuR has also been shown to bind to and stabilize the mRNA of

cytoprotective genes such as p53 under times of stress (e.g., UV irradiation) (166). Bioinformatics analyses identify multiple AU-rich elements within the 3'UTR for Srxn1 (see http://nibiru.tbi.univie.ac.at/AREsite2/welcome). Hence, it is not unreasonable to hypothesize that HuR or other RNA binding proteins may alter Srxn1 expression and that disrupted translocation in AhR-deficient cells may alter this pattern of expression. Perhaps a set of experiments designed to elucidate the relationship between Srxn1 and RNA binding proteins such as HuR in AhR-expressing and -deficient cells exposed to CSE could be performed. Indeed, a tool such as CLIP-seq (cross-linking immunoprecipitation-sequencing) technology could be useful for this purpose. In such a technique, RNA-protein complexes are extracted, and the bound RNA content from a protein (*e.g.*, HuR) is assessed by qRT-PCR. If such an HuR and Srxn1 mRNA interaction took place, alterations in Srxn1 expression in response to AhR activity or CSE exposure could lead to a possible mechanism by which the AhR regulates Srxn1. However, although a possible nongenomic mechanism for AhR control over Srxn1 expression could be elucidated through either of these two plausible scenarios, it is still unclear how the AhR is able to regulate either miRNA expression or RNA binding protein translocation.

Aside from the mechanism whereby the AhR regulates Srxn1 expression in mouse lung fibroblasts, it is also worth investigating the degree to which Srxn1 is important in AhR-mediated antioxidant defense. Given the robust and significant elevation in oxidative stress between AhR-deficient fibroblasts and wild-type, and given that $AhR^{DBD/DBD}$ showed little more oxidative stress levels over controls when treated with CSE, we can surmise that nongenomic activities for the AhR are necessary for mediating sufficient antioxidant defense. The possibility exists that other enzymatic mechanisms of antioxidant defense (*e.g.*, thioredoxins) may also be regulated in an AhR-dependent manner. It is, therefore, useful to clarify the cytoprotective role of Srxn1 in CSE-

challenged cells. This could be accomplished with Srxn1 siRNA knockdown in AhR-expressing cells; conversely, Srxn1 knock-in in AhR-deficient cells could also elucidate the protective nature of Srxn1 against CSE-induced apoptosis and oxidative stress. Srxn1 is also needed for the reactivation of hyperoxidized Prx (65). Although our gene array had not identified changes in mRNA levels of Prx and related genes, it may be important to establish the redox status of Prx in our cell model to elucidate the mechanism by which Srxn1 protects fibroblasts from CSE in an AhR-dependent manner.

As of now, a working model for AhR regulation of antioxidant expression has been established (Fig 4.1). In response to cigarette smoke-induced ROS, the AhR activates Nqo1 transcription and translation in a DRE-dependent manner. However, in mouse lung fibroblasts, Srxn1 seems to be key in regulating antioxidant defense. Until further investigation, the mechanism by which the AhR regulates Srxn1 remains a mystery.

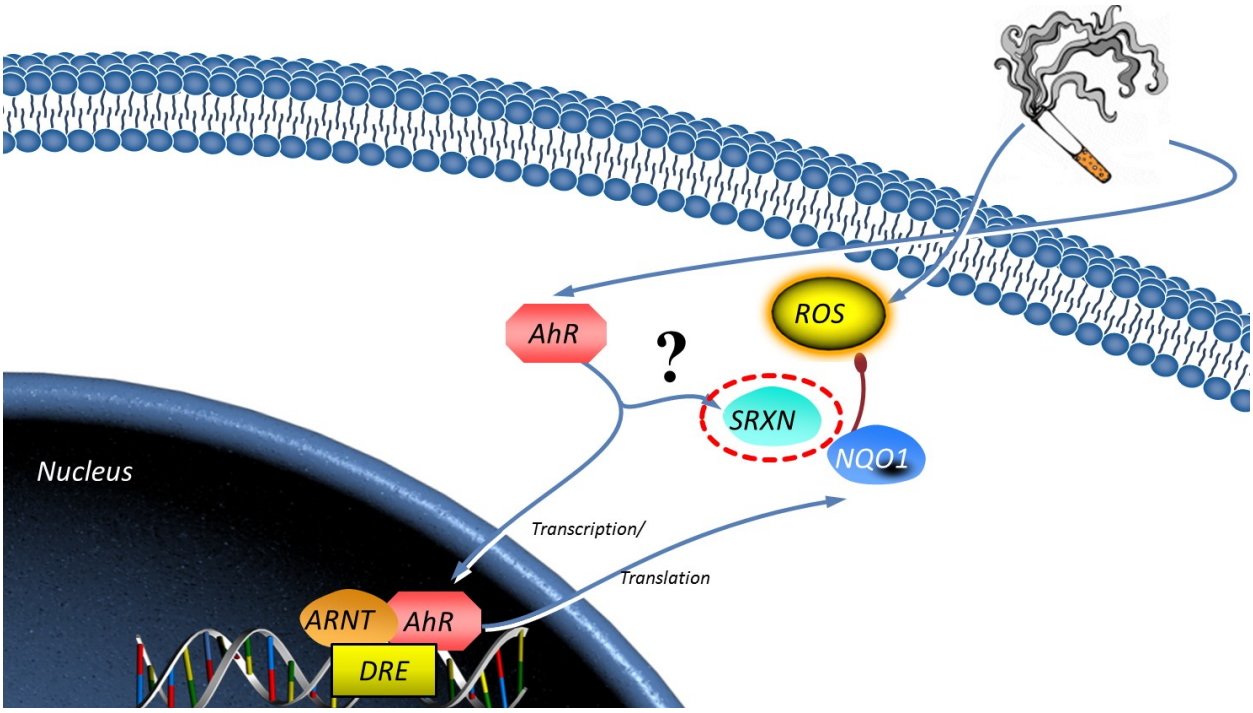


Figure 4.1. Working model of "The AhR Mediated Antioxidant Pathway" CSE produces ROS and activates the AhR in a bimodal manner. The AhR translocates to the nucleus where it binds with ARNT to the DRE, transcribing for antioxidant genes such as Nqo1. Srxn1 is regulated by the AhR in a nongenomic and DRE-independent manner. The mechanism for this regulation is yet unknown. Both Nqo1 and Srxn1 protect against CSE-induced ROS and oxidative stress.

4.2 The AhR in Human Health and Disease

Heightened oxidative stress and apoptosis are pathological hallmarks of COPD. Although 80-90% of COPD cases are caused by chronic tobacco smoke usage, only 15% of smokers develop COPD (15,158). This indicates that a genetic predisposition toward COPD is likely. Genomic and proteomic mechanisms for decreased defense against cigarette smoke-induced COPD have been

investigated and include genes involved in inflammation, aging, and oxidative stress. Transcription factors that function in tandem to the AhR such as Nrf2 or members of the NF-κB family, have been investigated in relation to human and *in vivo* models for COPD pathogenesis (21,30,66,151,167,168). We have reported for the first time that the AhR protein expression is significantly reduced in fibroblasts from COPD patients compared to Normal subjects (non-smokers) and At Risk subjects (smokers but without disease). We found that cells from COPD patients also displayed significantly lowered AhR activity and Nqo1 expression. Additionally, there was no significant increase in Srxn1 levels in COPD lung fibroblasts exposed to CSE, suggesting decreased antioxidant capacity in these cells. Given that oxidative stress is a key component of COPD pathogenesis and taken together with our findings on altered AhR expression in COPD-derived cells, it is conceivable that decreased AhR expression in COPD patients may be a key biological determinant for disease progression and status.

4.2.1 A Regulatory Role for the AhR in Metabolism, Insulin Resistance and Neurodegeneration?

Although COPD is primarily a constellation of syndromes relating to lung pathophysiology, metabolic syndrome, insulin resistance and signs of accelerated aging also often accompany COPD as comorbidities (54,169,170). It would be interesting to explore the relationship between the AhR, inflammation, oxidative stress, and insulin resistance, given their putative correlation. Insulin resistance of neurons has recently been linked to Alzheimer's disease, with glycogen synthase kinase-3β (GSK-3β) dysregulation serving as a pathogenic mechanism that precedes the hallmark biomarkers for neurodegeneration (171–174). Interestingly, there seems to be some possible crosstalk between the AhR and GSK-3β. TCDD exposure was found to lead to the phosphorylation

and inactivation of GSK-3β in HAPI microglial cells alongside the production of ROS and the inflammatory cytokine TNF-α (175). GSK-3β, purported to be involved with peripheral insulin resistance (176,177), is not known to directly interact with the AhR. Numerous recent articles have indicated that potent AhR agonists including TCDD and methylated indirubin have an inhibitory effect on GSK-3β (178), yet it is still unclear as to whether these two molecular factors participate in any biochemical axis, neuronal or otherwise. In the brain, activity of the AhR has been most often described as having the properties of a "double-edged sword," with AhR deficiency leading to deleterious alterations in hippocampal neurogenesis and overactivation (by ligands such as TCDD) leading to neurotoxicity (179,180). Nevertheless, nonclassical modulation of the AhR by the *Brassica* family-derived phytonutrient diindolylmethane (DIM) promoted cytoprotection against hypoxia-induced cell death in mouse neuronal cells (181). Srxn1 has also been shown to promote cytoprotective effects against oxygen-glucose deprivation-induced oxidative stress in Wistar rat cerebral astrocytes (160). Srxn1 silencing resulted in decreased SOD, GSH, and cell viability. DIM, which has been shown to both modulate AhR transcriptional activity as well as activate the Nrf2-ARE pathway, could then hypothetically drive Srxn1 expression (181,182). DIM was shown to protect primary murine neuronal cells from hypoxia-induced oxidative stress and apoptosis by impairing AhR-DRE activity (181). These findings are consistent with our model that nongenomic regulation of the AhR, rather than classical AhR-DRE signaling, is what may mediate antioxidant and Srxn1 activity. Additional phytonutrients from the *Brassica* family of vegetables such as indolo[3,2-b]carbazole have also been shown to reduce oxidative stress in an AhR-dependent manner in the Caco-2 cell line (183). Given that we have discovered decreased Srxn1 in AhR-deficient mouse lung fibroblasts in response to CSE, it would be fascinating to explore AhR-Srxn1

mediated antioxidant defense in models of neuroprotection and neurodegenerative disease. Research on phytonutrient-induced Srxn1 expression by the AhR is also warranted.

4.2.2 The AhR as a Central Regulator of Gut Inflammation: similarities to COPD

The AhR may play a role in inflammatory bowel disease (IBD). IBD is a category of diseases that include Crohn's disease (CD) and ulcerative colitis (UC). Interestingly, both IBD and COPD appear to share numerous pathophysiological similarities including epithelial barrier dysfunction, exaggerated by protease imbalance, dysbiosis, and inappropriate immune response to intestinal/lung microbiota and systemic inflammation. Cigarette smoke is a known risk factor for IBD, especially in CD where tobacco consumption is associated with a 3-fold increase in pathogenesis (184). IBD patients often also experience subclinical pulmonary inflammation that may contribute to COPD disease progression (185). In both COPD and IBD, there is an elevation in the acute phase cytokine IL-6, associated with apoptosis and tissue damage (186–189). Additionally, both IL-6 and the AhR contribute to the development of Th17 cells (190). Enhanced production of Th17 cell-related cytokines including IL-17 may propagate IBD as well as COPD (184). Given our findings on AhR expression in COPD patients, the function of the AhR may be important to understanding IBD pathogenesis. Interestingly, protein levels of the AhR are reduced in IBD, and activation of the AhR leads to expression of IL-22, which attenuates gut inflammation (191). Activation of the AhR has been demonstrated to ameliorate dextran sodium sulfate (DSS)-induced colitis model of UC in mice (192). The activity of the AhR in IBD may also be linked to disease progression through the intestinal microbiome. It is known that numerous bacterial species synthesize tryptophan metabolites for microbial signaling that may act as putative AhR activators

(193). It is also known that a variety of commensal or probiotic bacteria may serve to halt IBD disease progression and reduce inflammation, a mechanism that may rely on AhR activity (194,195). The probiotic organism *Lactobacillus delbreuckii subsp. bulgaricus* OLL1181 has been shown to inhibit DSS-induced colitis through AhR activation by oral administration in mice. Antagonism of the AhR by α-naphthoflavone (αNF) prevented the anti-inflammatory effect of treatment with *L. bulgaricus* OLL1181 (196). The existence of a putative gut-lung axis has led researchers to explore the use of probiotic species for the dietary treatment of lung disease. COPD-related comorbidities may be managed by treatment with certain *Lactobacillus* strains (194).

4.3 Concluding Remarks

Research into the AhR, which for the past three decades has been explored as the "dioxin receptor," is undergoing a renaissance as investigators examine novel endogenous roles for this ubiquitously-expressed cytosolic transcription factor. We have for the first time discovered that the AhR regulates the expression of the antioxidant enzyme Srxn1 in response to cigarette smoke, and speculate upon its role in cytoprotection and abrogating oxidative stress in our model. We find that both the control of Srxn1 and Nqo1 by the AhR is independent of Nrf2, the transcription factor sometimes termed "the master regulator of antioxidant defense." As further research is conducted into the AhR along with its promiscuous multitude of cytosolic binding partners and putative nongenomic activity, this author predicts new and fascinating discoveries in the realm of chronic diseases that pose global health challenges today.

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