Functional Deficiency of Aryl Hydrocarbon Receptor Augments Oxygen Toxicity-Induced Alveolar Simplification in Newborn Mice

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Abstract

Hyperoxia contributes to the development of bronchopulmonary dysplasia (BPD) in premature infants. New BPD is characterized as having alveolar simplification. We reported previously that aryl hydrocarbon receptor (AhR) deficiency increased susceptibility to hyperoxic lung injury in adult mice, and this was associated with decreased expression of cytochrome P450 1A enzymes and increased lung inflammation. Whether AhR protects newborn mice against hyperoxia-induced alveolar simplification is unknown. Thus, we tested the hypothesis that decreased activation of the pulmonary AhR augments hyperoxia-induced alveolar simplification and lung inflammation in newborn mice. Experimental groups included one-day old wild type (WT) and AhR dysfunctional (AhRd) mice exposed to 21% O_2 (air) or 85% O_2 (hyperoxia) for 14 d. Exposure of newborn WT mice to hyperoxia resulted in increased protein, enzyme and mRNA expression of the AhR-regulated lung cytochrome P450 1A1, NAD(P)H quinone oxidoreductase-1, and microsomal glutathione S-transferase 1 enzymes, suggesting that hyperoxia increases activation of the pulmonary AhR. On the other hand, in the AhRd mice, hyperoxia induced the AhR-regulated enzymes to a lesser extent probably due to the dysfunctional AhR in these mice. Alveolar simplification and lung inflammation was increased in mice exposed to hyperoxia compared to those exposed to air, and AhRd mice were more susceptible to hyperoxia-induced alveolar simplification and lung inflammation compared to WT mice. These findings suggest that decreased activation of the pulmonary AhR in newborn AhRd mice augments hyperoxia-induced alveolar simplification and lung inflammation in these mice.

Keywords

Aryl hydrocarbon receptor; hyperoxia; alveolar simplification; lung inflammation

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Conflict of Interest Statement
The authors declare that there are no conflicts of interest.

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease whose primary structural and functional defect is alveolar simplification (Husain *et al.*, 1998; Jobe, 1999). Despite significant improvements in neonatal intensive care management of premature infants, BPD remains the most prevalent complication in these patients affecting approximately 52% of the extremely low birth weight infants (< 1000 g birthweight) (Natarajan *et al.*, 2012). Infants developing BPD are more likely to have long-term pulmonary problems, increased re-hospitalizations during the first year of life, and abnormal neurodevelopment compared to infants of similar birth weights and gestation that do not develop BPD (Short *et al.*, 2003; Fanaroff *et al.*, 2007). Thus, there is an urgent need for improved therapies in the prevention and treatment of BPD.

Supplemental oxygen is commonly administered as an important and life-saving measure in patients with impaired lung function. Although delivery of enriched oxygen relieves the immediate life-threatening consequences of hypoxemia, it may also exacerbate lung injury (Thiel *et al.*, 2005). Excessive oxygen exposure and lung stretching leads to increased reactive oxygen species (ROS) production and expression of proinflammatory cytokines (Jobe *et al.*, 2008). ROS react with nearby molecules (e.g., protein, lipids, DNA, and RNA) and modify their structure and function (Bhandari, 2010), and alter signal transduction pathways all of which frequently results in chronic pulmonary toxicity such as BPD. In addition, the antioxidant defense system develops late in gestation, making preterm neonates highly susceptible to oxidative stress (Vina *et al.*, 1995; Asikainen and White, 2005). Evidence implicates oxidative stress (Bhandari, 2010; Saugstad, 2010) and inflammation (Wright and Kirpalani, 2011) as major contributors to the development of BPD and its sequelae. However, the molecular mechanisms by which oxidative stress and inflammation causes BPD remain poorly understood.

The aryl hydrocarbon receptor (AhR) is a member of basic-helix-loop-helix/PER-ARNT-SIM family of transcriptional regulators (Burbach *et al.*, 1992). The AhR is expressed in all mouse tissues (Abbott *et al.*, 1995), and in humans, AhR is highly expressed in the lungs, thymus, kidney, and liver (Tirona and Kim, 2005). AhR activation results in the translocation of the cytosolic AhR to the nucleus, where it dimerizes with the AhR nuclear translocator to form a heterodimeric transcription factor. The heterodimeric transcription factor activates the transcription of many phase I and phase II detoxification enzymes such as cytochrome P450 (CYP) 1A1, CYP1A2, glutathione S-transferase-α (GST-α), NAD(P)H quinone oxidoreductase-1 (NQO1), UDP glucuronosyl transferase (UDPGT), and aldehyde dehydrogenase (ALDH), which are encoded by the Ah gene locus (Rushmore *et al.*, 1990; Favreau and Pickett, 1991; Emi *et al.*, 1996). AhR is of particular interest to toxicologists and extensive research has been conducted on its role in the bioactivation of polycyclic and aromatic hydrocarbons leading to carcinogenesis (Nebert *et al.*, 2004). Transgenic mice with AhR deficiencies have provided insight into the potential role(s) that AhR might play in normal physiological homeostasis (Bock and Kohle, 2009; Fujii-Kuriyama and Kawajiri, 2010). We reported earlier that adult mice deficient in AhR are more susceptible to hyperoxic lung injury compared to wild type controls and this phenomenon was associated with marked decreases in the expression of pulmonary and hepatic CYP1A subfamily of enzymes that have been reported to detoxify lipid hydroperoxides generated by reactive oxygen species (ROS) (Couroucli *et al.*, 2002; Jiang *et al.*, 2004). Recently, the AhR has been shown to attenuate tobacco smoke-induced inflammation in the lungs (Thatcher *et al.*, 2007; Baglole *et al.*, 2008), suggesting that AhR is a suppressor of lung inflammation. However, whether AhR attenuates hyperoxia-induced inflammation and alveolar simplification in the newborn lungs are unknown, and the current study was done to address this gap. Hence, the objective of our study was to elucidate the mechanistic role of AhR in

hyperoxia-induced alveolar simplification and lung inflammation in newborn mice. We pursued our objective by testing the hypothesis that decreased activation of the pulmonary AhR augments hyperoxia-induced alveolar simplification and lung inflammation in newborn mice.

Materials and Methods

Animals

This study was approved and conducted in strict accordance with the federal guidelines for the humane care and use of laboratory animals by the Institutional Animal Care and Use Committee of Baylor College of Medicine (Protocol number: AN-5631). C57BL6/J wild type (WT) and aryl hydrocarbon dysfunctional B6.D2N-Ahr^d/J (AhRd) mice were obtained from Charles River laboratories (Wilmington) and Jackson laboratories (Bar Harbor, ME), respectively. Dr. Daniel Nebert (University of Cincinnati, Cincinnati, OH) initially backcrossed Ahr^d allele from DBA/2N onto C57BL/6N via a backcross-intercross breeding scheme and transferred this congenic to Dr. Alan Poland (University of Wisconsin, Madison, WI) at generation N13, who then backcrossed the Ahr^d allele onto C57BL/6J, again via a backcross-intercross breeding scheme. The resulting homozygotes at or beyond generation N17 were maintained at the Jackson laboratory by sibling intercross. The AhR dysfunction in AhRd mice is due to decreased affinity of the AhR^d receptor for its ligand. There are 10 nucleotide differences in the coding regions between the AhR^b allele present in WT mice and the AhR^d allele present in AhRd mice. The structural changes in the AhR^d receptor associated with these nucleotide differences is thought to be responsible for the differential agonist affinity between the AhR^b and AhR^d receptors (Chang *et al.*, 1993). We maintained active colonies of WT and AhRd mice by breeding them in the animal facility at Texas Children's Hospital's Feigin Center. Time pregnant WT and AhRd mice raised in our animal facility were used for the experiments.

Exposure

Within 12 h of birth, pups from multiple litters were pooled before being randomly and equally redistributed to the dams, following which they were immediately exposed to either 21% O_2 (air) or 85% O_2 (hyperoxia) for 14 d as described earlier (Park *et al.*, 2007). The dams were rotated between air- and hyperoxia-exposed litters every 24 h to prevent oxygen toxicity in the dams and to eliminate maternal effects between the groups. Oxygen exposures were conducted in Plexiglas chambers, into which O_2 was delivered through an oxygen blender to achieve a constant level of 85% O_2. Animals were monitored every 12 h for evidence of adverse lung symptoms or mortality.

Analyses of the pulmonary AhR activation

It is reported that functional activation of the AhR results in the expression of many phase I and II enzymes. So, we determined the functional activation of the pulmonary AhR by analyzing the expression of pulmonary CYP1A1 (phase I), and NQO1 and microsomal glutathione S-transferase 1 (MGST1) (phase II) enzymes.

Lung tissue preparations for analyses of the AhR activation

Following exposure, animals were euthanized with i.p. injections of 200 mg/kg of sodium pentobarbital and their lungs (n=6/group) were stored at − 80°C for isolation of total RNA. The lungs from a separate set of animals (n=10/group) were snap frozen in liquid nitrogen for subsequent isolation of nuclear and cytosolic proteins.

Preparation of nuclear and cytosolic protein

A mortar and pestle was used to homogenize the lung tissue in a buffer containing 50mM Tris-HCL (pH 7.5), 0.5M KCL, 1M MgCL, and 0.5M EDTA. The homogenate was centrifuged at 2400 g for 5 min at 4°C. The supernatant (cytoplasmic fraction) was stored at − 80°C. The pellet was resuspended in a lysis buffer containing 50mM Tris-HCL (pH 7.5), 2.1M NaCL, 1M MgCL, 0.5M EDTA, and 25% sucrose, incubated on ice for 20 min, and centrifuged at 19000 g at 4°C for 5 min. The resulting supernatant (nuclear fraction) was stored at − 80°C until further use.

Enzyme assays

CYP1A1 and NQO1 enzyme activities were measured in the cytosolic fraction according to the published protocols (Benson *et al.*, 1980; Preusch *et al.*, 1991; Moorthy *et al.*, 2000). GST enzyme activity was quantified by using a GST assay kit according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO; CS0410).

Western blot assays

Ten or 20 μg of lung cytosolic protein extracts were separated by 10% SDS-polyacrylamide gel electrophoresis for detection of CYP1A1, NQO1, and MGST1 apoproteins, and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with the following primary antibodies: anti-CYP1A1 antibody (gift from P.E. Thomas, Rutgers University, Piscataway, NJ, USA; dilution 1:1500), anti-NQO1 antibody (Santa Cruz Biotechnologies; sc-16464, dilution 1:500), anti-MGST1 antibody (Santa Cruz; sc-17003, dilution 1:500) and anti-β-actin antibody (Sigma-Aldrich; A5316, dilution 1:5000). The primary antibodies were detected by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive bands were detected by chemiluminescence methods and the band density was analyzed by Kodak 1D 3.6 imaging software (Eastman Kodak Co., Rochester, NY, USA).

Quantitative real-time RT-PCR assays

Total RNA extracted from frozen lung tissues using Trizol reagent (Invitrogen) were treated with RQ1 RNase-free DNase I (Promega, Madison, WI) to eliminate genomic DNA contamination. RNA (50 ng), isolated as above, was subjected to one-step real-time quantitative TaqMan RT-PCR. Gene specific primers (CYP1A1-Mm00487218_m1; NQO1-Mm01253561_m1; MGST1-Mm00498294_m1; and 18S-Hs99999901_s1) in the presence of TaqMan reverse transcription reagents were used to reverse transcribe RNA, and TaqMan Gene Expression probes and TaqMan Universal PCR Master Mix (Applied Biosystems) were used for PCR amplification. The 18S was used as the reference gene. After an RT hold for 30 min at 48°C, the samples were denatured at 95°C for 10 min. The thermal cycling step was for 40 cycles at 95°C for 15s and 40 cycles at 60°C for 1 min. The $\Delta\Delta C_t$ method was used to calculate the fold change in mRNA expression (Jiang *et al.*, 2004).

Analyses of alveolarization

Tissue preparation for lung morphometry and immunohistochemistry—After completion of gas exposures, a subset of pups were euthanized and their lungs were inflated and fixed via the trachea with 10% formalin at 25 cm H_2O pressure for at least 10 min. Serial five-micrometer sections of the paraffin embedded lungs were obtained perpendicular to the lung base (apical-basal axis) to attain portions of all the lobes of both the lungs for analysis. A systematic, uniform, random sampling principle (Hsia *et al.*, 2010) was used to evaluate the sections for lung morphometry.

Lung morphometry—Alveolar development on selected mice (n=6/group) was evaluated by radial alveolar counts (RAC) and mean linear intercepts (MLI). The observers performing the measurements were masked to the slide identity. 1. *Radial alveolar counts:* RAC was determined as described by Cooney and Thurlbeck (Cooney and Thurlbeck, 1982). RAC measurements were made by dropping a perpendicular line from the center of a respiratory bronchiole to the edge of the septum or pleura and counting the number of alveoli traversed by this line. 2. *Mean linear intercepts:* MLIs were assessed as described previously (van Eijl *et al.*, 2011). Briefly, grids of horizontal and vertical lines were superimposed on an image and the number of times the lines intersected with the tissue was counted. The total length of the grid lines was then divided by the number of intersections, to provide the mean linear intercept in μm. Photographs from at least 10 random nonoverlapping lung fields (10x magnification) were taken from each animal for RAC and MLI measurements.

Analyses of lung inflammation—Lung inflammation was assessed by 1. *Immunostaining for macrophages:* Deparaffinized lung sections (5 μM) were stained with rat anti-mouse Mac-3 antibody (553322, BD Pharmingen; dilution 1: 750), followed by staining with the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The number of macrophages in the alveolar air spaces was counted from at least 10 random nonoverlapping lung fields (x400 magnification) per animal (n=6/group). The observers performing the macrophage counts were masked to the slide identity. 2. *Monocyte chemoattractant protein 1 (MCP-1) mRNA expression:* Total RNA extracted from lung tissues was used to quantify MCP-1 mRNA expression by real-time RT-PCR using a specific primer (MCP1-Mm00441242_m1, Applied Biosystems Inc., Foster City, CA) as described in analyses of CYP1A1, NQO1, and MGST1 mRNA expression.

Statistical Analyses

Statistical analyses were performed using SPSS 19.0. Data are expressed as means ± SEM. For the mRNA analysis, the fold change in mRNA expression was calculated by normalization to the air-exposed animals. The effects of genotype, exposure and their associated interactions for the outcome variables were assessed using ANOVA techniques. Multiple comparison testing by the posthoc Bonferroni test was performed if statistical significance of either variable or interaction was noted by ANOVA. The results were considered statistically significant if the p value of the posthoc Bonferroni test was less than 0.05.

Results

In this study, we investigated the role of AhR in hyperoxia-induced alveolar simplification and inflammation in newborn mice.

Hyperoxia Enhances Pulmonary CYP1A1, NQO1, and MGST1 mRNA Expression in WT Mice

To examine the effects of hyperoxia on functional activation of the AhR, we determined the expression of pulmonary CYP1A1, NQO1 and MGST1 mRNA, which are the phase I and II enzymes that are induced upon activation of the AhR. Quantification of mRNA levels by RT-PCR analysis in WT mice demonstrated that exposure to hyperoxia increased pulmonary CYP1A1 (Fig 1A), NQO1 (Fig 1B), and MGST1 (Fig 1C) mRNA levels by approximately 3-, 4-, 2.5-fold, respectively, when compared to corresponding air-breathing animals.

Hyperoxia Enhances Pulmonary CYP1A1, NQO1, and MGST1 Protein Expression in WT Mice

Next, we determined the effect of hyperoxia on pulmonary CYP1A1, NQO1, and MGST1 apoprotein expression by western blotting. The pulmonary CYP1A1 (Fig 2A, B), NQO1 (Fig 2C, D), and MGST1 (Fig 2E, F) apoprotein expression was significantly increased in WT mice exposed to 14 d of hyperoxia compared to air-breathing animals. This was consistent with the effects of hyperoxia on the mRNA expression of the AhR-regulated phase I and II enzymes.

Hyperoxia Enhances Pulmonary CYP1A1, NQO1, and GST Enzyme Activities in WT Mice

Finally, we examined the effects of hyperoxia on the pulmonary CYP1A1, NQO1 and MGST1 enzyme activity levels. WT mice exposed to 14 d of hyperoxia had significantly increased AhR-regulated phase I CYP1A1 enzyme activity (Fig 3A), and phase II NQO1 (Fig 3B) and GST (Fig 3C) enzyme activities compared to air-breathing WT mice.

Hyperoxia-Induced Expression of Pulmonary CYP1A1, NQO1, and MGST1 Enzymes were Attenuated in AhRd mice

To validate that AhR was dysfunctional in AhRd mice, we determined the effects of hyperoxia on the expression of pulmonary CYP1A1, NQO1, and MGST1 enzymes in AhRd mice. Not surprisingly, hyperoxia-induced expression of pulmonary CYP1A1, NQO1, and MGST1 enzymes were attenuated at the mRNA (Fig 1), protein (Fig 2), and enzyme (Fig 3) levels in AhRd mice compared to similarly exposed WT mice.

AhRd Mice had Increased Hyperoxia-Induced Alveolar Simplification

To investigate the mechanism(s) by which hyperoxia contributes to alveolar simplification, we studied the impact of AhR on alveolar simplification by exposing newborn WT and AhRD mice to hyperoxia. Alveolar simplification was determined by RAC and MLI. Exposure of WT and AhRD mice to 14 d of hyperoxia resulted in a significant decrease in RAC (Fig 4B, D, E) indicating that their alveoli were fewer in number compared to corresponding air-breathing animals (Fig 4A, C, E). In addition, hyperoxia-exposed WT and AhRd mice had significant increases in MLI (Fig 4B, D, F) indicating that their alveoli were also larger compared to corresponding air-breathing animals (Fig 4A, C, F). Interestingly, hyperoxia-induced alveolar simplification was significantly increased in AhRd mice compared to similarly exposed WT mice (Fig 4B, D, E, F). Statistical analyses indicated a significant two-way interaction between the genotype and hyperoxia exposure on alveolarization. In air-breathing animals, there was no significant difference in alveolarization between WT and AhRd mice (Fig 4A, C, E, F).

AhRd Mice had Increased Hyperoxia-Induced Lung Inflammation

We performed real-time RT-PCR analysis of lung MCP-1 mRNA, and immunohistochemistry on fixed lung sections using anti-Mac-3 antibodies to ascertain if AhR altered hyperoxia-induced lung inflammatory response. Real-time RT-PCR analysis and the immunohistochemistry study revealed that hyperoxia increased MCP-1 mRNA expression (Fig 5F) and accumulation of macrophages (Fig 5B, D, E) in the lungs of both WT and AhRd mice. However, the effects of hyperoxia-induced lung MCP-1 expression and macrophage influx were augmented in AhRd mice (Fig 5B, D, E, F) compared to similarly exposed WT mice. Statistical analyses indicated a significant two-way interaction between the genotype and hyperoxia exposure on lung inflammation. In air-breathing animals, there was no significant difference in lung MCP-1 expression and macrophage counts between WT and AhRd mice (Fig 5A, C, E, F).

Discussion

This study demonstrates that hyperoxia exposed AhR deficient newborn mice have an increased susceptibility to alveolar simplification, which is associated with an augmented lung inflammatory response. In newborn mice exposed to hyperoxia, alveolar simplification and lung inflammation correlated inversely with the activation of the pulmonary AhR and the expression of its downstream target genes, CYP1A1, NQO1, and MGST1.

To investigate the molecular mechanisms associated with hyperoxia-induced alveolar simplification, we examined the lungs of hyperoxia-exposed newborn AhRd mice. The AhR is a versatile transcription factor that has important physiological functions in addition to its widely established role in induction of a battery of genes involved in the metabolism of xenobiotics. Studies from our laboratory and others have reported that AhR may be a crucial regulator of oxidant stress and inflammation through induction of several detoxifying phase I and II enzymes or via cross talk with other signal transduction pathways. In adult mice, AhR deficiency has been shown to be associated with increased hyperoxic lung injury (Couroucli *et al.*, 2002; Jiang *et al.*, 2004). Furthermore, we demonstrated that activation of AhR by omeprazole in adult mice (Shivanna *et al.*, 2011b) and in the adult human lung-derived H441 cells (Shivanna *et al.*, 2011a) attenuates hyperoxic injury. Whether AhR and its target genes are responsible for protecting newborn lung against hyperoxic lung injury is unknown. The goal of this study thus was to determine whether AhR was involved in hyperoxia-induced alveolar simplification and lung inflammation in newborn mice.

Initially, we studied the interaction between hyperoxia and AhR. Functional activation of the AhR results in transcriptional activation of various target genes referred to as the AhR gene battery, some of which includes CYP1A1, NQO1, and MGST1. Hence, we analyzed the expression of pulmonary CYP1A1, NQO1, and MGST1 to determine the activation of the pulmonary AhR. Strikingly, hyperoxia activated pulmonary AhR as evident by increased expression of pulmonary CYP1A1, NQO1, and MGST1 in WT mice. However, we did notice a discrepancy between CYP1A1 activity and CYP1A1 mRNA and protein levels, which we believe may be due to the interference in the ethoxyresorufin deethylase activity by other heme proteins. Enhanced pulmonary CYP1A1, NQO1, and MGST1 mRNA expression that is seen in parallel with corresponding increases in enzyme activities and apoprotein contents provides evidence that hyperoxia induces these enzymes by a transcriptional mechanism. On the contrary, hyperoxia had a minimal induction of pulmonary CYP1A1, NQO1, and MGST1 genes in newborn AhRd mice. This suggests that these enzymes are downstream target molecules that are transcriptionally activated by the pulmonary AhR. However, the molecular mechanisms by which hyperoxia activate pulmonary AhR remain unknown at the present time.

Hyperoxia-induced expression of the phase I and II enzymes have been observed by several other investigators both in adult (Moorthy *et al.*, 1997; Cho *et al.*, 2002; Couroucli *et al.*, 2002; Jiang *et al.*, 2004) and newborn rodents (McGrath-Morrow *et al.*, 2009; Couroucli *et al.*, 2011). This phenomenon might be a protective responsive to oxidative stress because of its striking resemblance to the effects of hyperoxia on the "classic" antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase (Ho *et al.*, 1996; Clerch, 2000). In our study, there were no significant differences in expression of these enzymes between air-breathing WT and AhRd mice. These findings suggest that the constitutive expression of these enzymes might be equally regulated by other transcription factors such as Nrf2 in addition to the AhR.

Next, we studied the effects of AhR on hyperoxia-induced alveolar simplification and lung inflammation. Hyperoxia is known to cause alveolar simplification both in preterm infants

(Husain *et al.*, 1998) and newborn mice (Warner *et al.*, 1998). A similar finding was noticed in our experimental animals exposed to hyperoxia for 2 weeks. However, the increased alveolar simplification noticed in hyperoxia-exposed AhRd mice compared to similarly exposed WT mice indicates that AhR protects newborn mice against hyperoxia-induced alveolar simplification.

Previous studies have demonstrated that ROS and inflammatory responses are the key mediators in the pathogenesis of hyperoxia-induced lung disorders such as BPD in preterm infants. MCP-1 is a CC chemokine that is increased in the bronchoalveolar lavage fluid and plasma of infants with oxidant injury who later develop BPD (Ambalavanan *et al.*, 2009). Likewise, lung macrophage infiltration is an important marker of a chronic inflammatory state that is associated with BPD (Clement *et al.*, 1988). Hence, we determined the lung MCP-1 expression and macrophage infiltration to determine the effects of AhR on hyperoxia-induced lung inflammatory response. Our observation of an increased hyperoxia-induced lung inflammatory response in AhRd mice suggests that AhR might mitigate alveolar simplification by decreasing lung inflammation, although we only demonstrated an association not necessarily a cause and effect relationship.

The molecular mechanisms by which the pulmonary AhR protects against hyperoxic lung injury remains poorly defined. Interestingly, we observed significant upregulation of pulmonary CYP1A1, NQO1, and MGST1 enzymes in WT mice compared to AhRd mice upon exposure to hyperoxia. The protective effects of CYP1A enzymes against hyperoxic lung injury in rodents have been extensively documented, as evidenced by 1) attenuation of hyperoxic lung injury in rodents treated with CYP1A inducers, β-naphthoflavone or 3-methylcholanthrene (Mansour *et al.*, 1988; Sinha *et al.*, 2005; Moorthy, 2008; Couroucli *et al.*, 2011); 2) potentiation of hyperoxic injury in rats treated with CYP1A inhibitor, 1-aminobenzotriazole (Moorthy *et al.*, 2000); 3) increased susceptibility of rodents deficient in genes for AhR (Couroucli *et al.*, 2002; Jiang *et al.*, 2004) to hyperoxic lung injury. In addition, the phase II enzymes such as NQO1 and MGST1 have been shown to protect cells and tissues against oxidant injury induced by various toxic chemicals (O'Brien, 1991; Rahman *et al.*, 1999; Johansson *et al.*, 2010) and oxygen (Cho *et al.*, 2002; Das *et al.*, 2006; McGrath-Morrow *et al.*, 2009). The protective mechanisms of these enzymes have been attributed to their ability to conjugate and excrete the reactive electrophiles and lipid peroxidation products generated by an oxidant injury (Cho *et al.*, 2002; Johansson *et al.*, 2010). AhR-mediated protection against hyperoxia-induced alveolar simplification may be attributed at least in part to these enzymes. It is important to note that AhR activation results in the induction of several detoxifying enzymes, which may be collectively more effective against an oxidant injury compared to the induction of a single enzyme. Furthermore, Baglole et al. (Baglole *et al.*, 2008) recently observed that AhR decreases inflammation in lung cells by regulating the transcription factor, NF-κb. We have also conducted experiments to investigate the interaction between AhR and NF-kB in hyperoxic conditions. Our preliminary experiments suggest that NFkB activity is increased in hyperoxia-exposed AhRd mice (data not shown). However, we need to confirm this finding by conducting additional experiments. It is thus possible the protective effects of the AhR might also be mediated in part by its interaction with NF-κb.

We recognize the limitations of our study. Firstly, the expression of the pulmonary AhR could not be precisely regulated because our experimental animals were not conditional knockout mice. However, these results provide a background to conduct studies using transgenic animals that target the developing lungs. Secondly, we have shown structural defects associated with altered gene expression, but not the functional consequences. In future, we will determine the functional correlate by performing lung function tests in these animals.

In summary, we demonstrate that decreased activation of the pulmonary AhR augments hyperoxia-induced alveolar simplification and increased lung inflammation in newborn mice. We propose that the protective effects of AhR may be due to the induction of a battery of phase I and II enzymes that have potential antioxidant and anti-inflammatory properties. It is important to note that even with extensive back cross breeding, the AhRd mice are still not similar to AhR-null mice and they have some residual AhR function. Therefore, we do expect the effects of AhR on alveolarization and the expression of phase I and II enzymes to be more remarkable when we use AhR-null mice in our future studies.

The etiology of BPD is multifactorial, and therefore, therapies targeting a single inflammatory or oxidative stress pathway have largely been unsuccessful in the management of BPD (Wright and Kirpalani, 2011). Recent studies including our present one suggests that AhR decreases lung inflammation, and upregulates phase I and II enzymes, which are known to have antioxidant properties. AhR can thus be used to improve current therapies of BPD in preterm infants because activation of pulmonary AhR would target more than one pathway and mitigate both inflammation and oxidant stress, which are significant in the development of BPD.

Acknowledgments

Abbreviations

BPD	Bronchopulmonary dysplasia
ROS	Reactive oxygen species
AhR	Aryl hydrocarbon receptor
CYP	Cytochrome P450
NQO1	NAD(P)H quinone oxidoreductase-1
MGST1	Microsomal glutathione S-transferase1
WT mice	C57BL6/J wild type mice
AhRd mice	Aryl hydrocarbon receptor dysfunctional mice
RAC	Radial alveolar count
MLI	Mean linear intercept
MCP-1	Monocyte chemoattractant protein-1
ANOVA	Analysis of variance

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Highlights

- AhR deficiency increases oxygen toxicity in newborn mice.

- Hyperoxia-exposed AhRd mice have decreased expression of CYP1A1, NQO1, and MGST1 enzymes.

- Functional deficiency of AhR increases hyperoxia-induced lung inflammation.

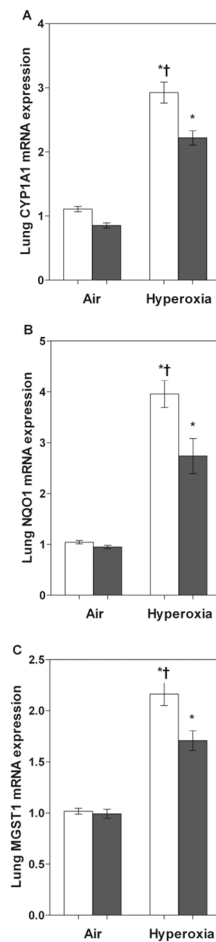- Functional deficiency of AhR increases hyperoxia-induced arrest in alveolarization.

Figure 1. Hyperoxia increases pulmonary CYP1A1, NQO1, and MGST 1 mRNA expression
Total RNA, isolated from the lungs of WT and AhRd mice exposed to air or hyperoxia for
14 d, was subjected to real-time RT-PCR analysis for CYP1A1 (A), NQO1 (B), and
MGST-1 (C) mRNA expression. Values are means ± S.E.M. from at least five individual
WT (open bars) and AhRd (closed bars) animals in air or hyperoxia groups. Two-way
ANOVA showed an effect of hyperoxia and genotype and a two-way interaction. Significant
differences between air-breathing and hyperoxia-exposed animals are indicated by *, $p <
0.05$. Significant differences between hyperoxia-exposed WT and AhRd mice are indicated
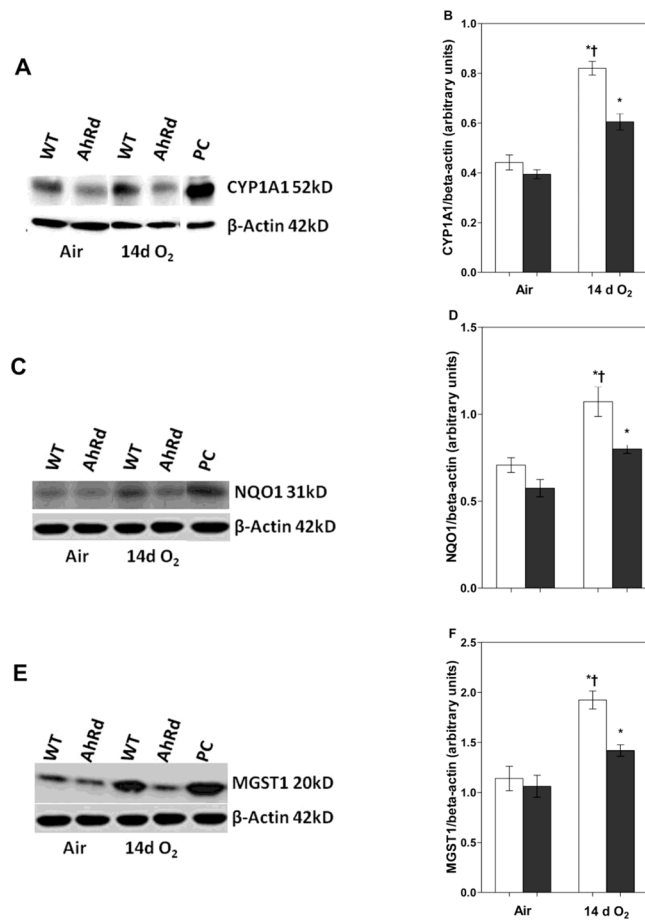by †, $p < 0.05$.

Figure 2. Hyperoxia increases pulmonary CYP1A1, NQO1, and MGST 1 protein expression
The lung homogenates of WT and AhRd mice exposed to air or hyperoxia for 14 d were separated into cytosolic and nuclear fractions. The cytosolic protein was subjected to western blotting using anti-CYP1A1, anti-NQO1, anti-MGST1, or anti-β-actin antibodies. A, C, and E are representative western blots showing the expression of CYP1A1, NQO1, and MGST1 proteins, respectively. PC: positive control (3-methylcholanthrene treated lungs). B, D, and F represent the relative amounts of CYP1A1, NQO1, and MGST1 proteins, respectively, that are normalized to β-actin. Values are means ± S.E.M. from at least five individual WT (open bars) and AhRd (closed bars) animals in air or hyperoxia groups. Two-way ANOVA showed an effect of hyperoxia and genotype and a two-way interaction. Significant differences between air-breathing and hyperoxia-exposed animals are indicated by *, $p < 0.05$. Significant differences between hyperoxia-exposed WT and AhRd mice are indicated by †, $p < 0.05$.
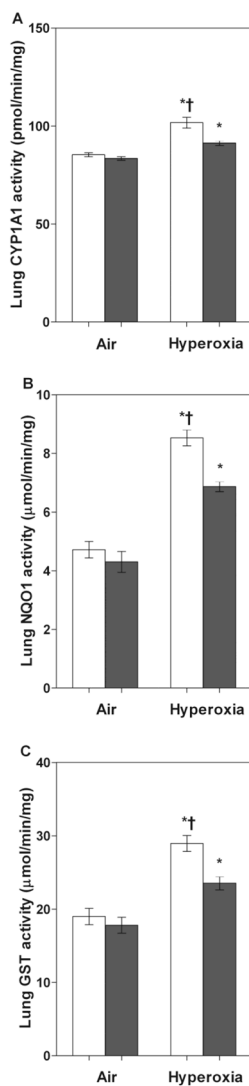
Figure 3. Hyperoxia increases pulmonary CYP1A1, NQO1, and GST enzyme activities
WT and AhRd mice were exposed to air or hyperoxia for 14 d, following which pulmonary CYP1A1 (A), NQO1 (B), and GST (C) enzyme activities were measured as described in materials and methods. Values are means ± S.E.M. from at least four individual WT (open bars) and AhRd (closed bars) animals in air or hyperoxia groups. Two-way ANOVA showed an effect of hyperoxia and genotype and a two-way interaction. Significant differences between air-breathing and hyperoxia-exposed animals are indicated by *, $p < 0.05$. Significant differences between hyperoxia-exposed WT and AhRd mice are indicated by †, $p < 0.05$.
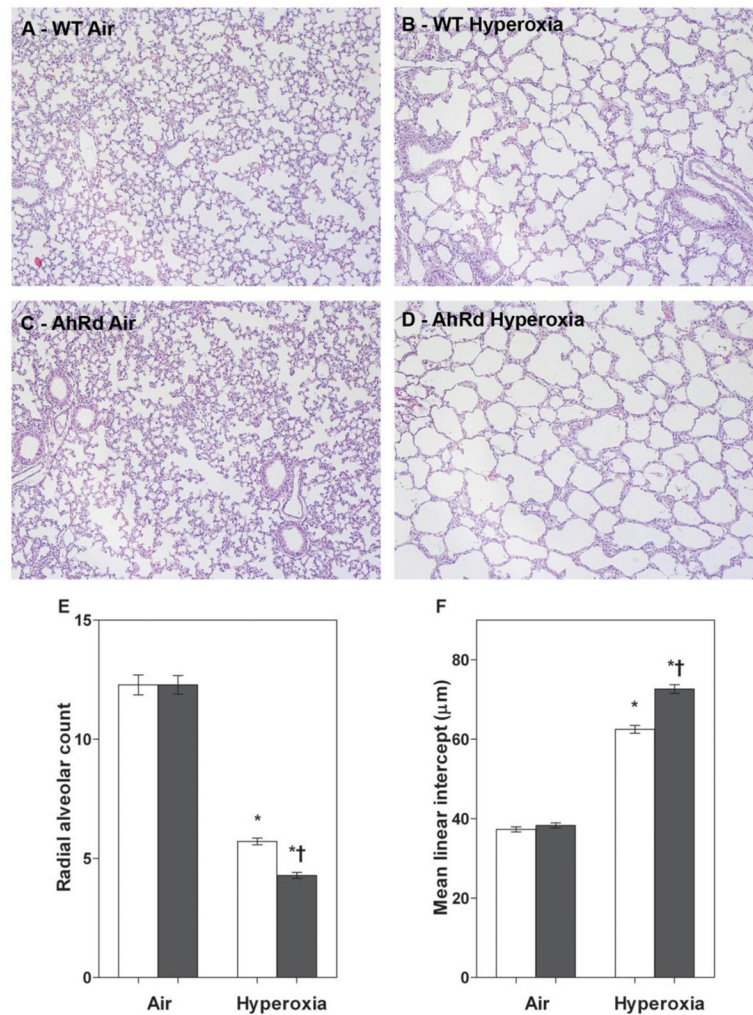
Figure 4. AhRd mice have increased hyperoxia-induced alveolar simplification
AD: Representative hematoxylin and eosin-stained lung sections obtained from WT (A and B) and AhRd (C and D) mice exposed to air (A and C) or hyperoxia (B and D) for 14 d. Alveolarization was quantified by radial alveolar count (E) and mean linear intercept (F). Values are means ± S.E.M. from at least five individual WT (open bars) and AhRd (closed bars) animals in air or hyperoxia groups. Two-way ANOVA showed an effect of hyperoxia and genotype and a two-way interaction. Significant differences between air-breathing and hyperoxia-exposed animals are indicated by *, $p < 0.05$. Significant differences between hyperoxia-exposed WT and AhRd mice are indicated by †, $p < 0.05$.
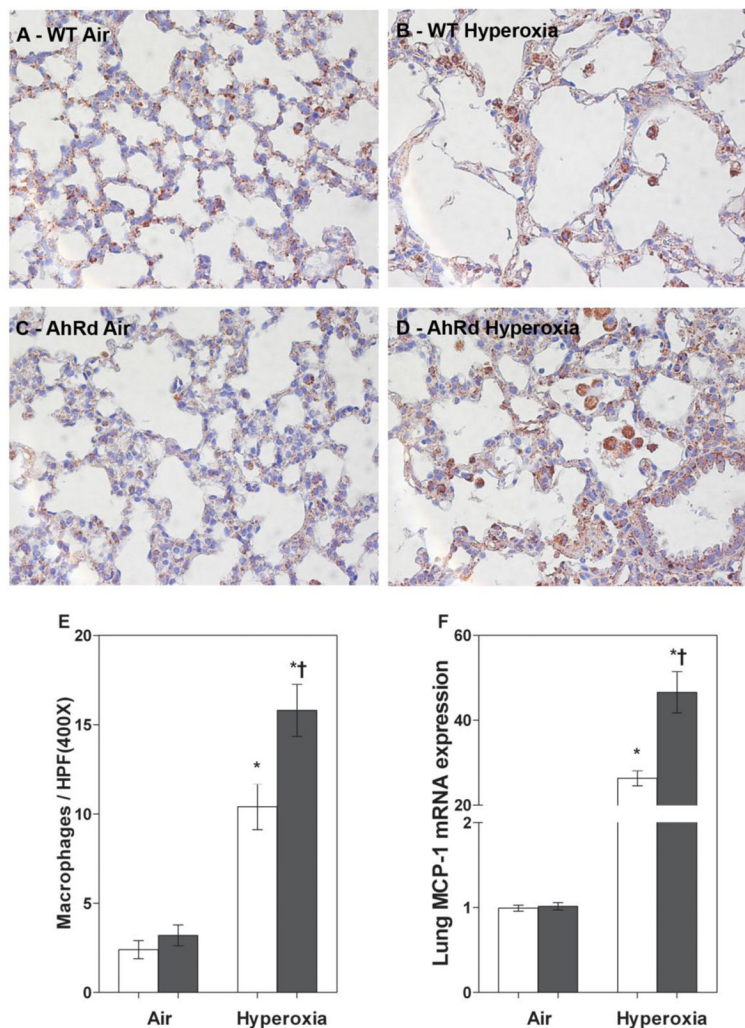
Figure 5. AhRd mice have increased hyperoxia-induced lung inflammation
A–D: Representative Mac 3 antibody immunostained lung sections obtained from WT (A and B) and AhRd (C and D) mice exposed to air (A and C) or hyperoxia (B and D) for 14 d. E. Quantitative analysis of macrophages per high power field. F. Real-time RT-PCR analysis showing the lung MCP-1 mRNA expression. Values are means ± S.E.M. from at least five individual WT (open bars) and AhRd (closed bars) animals in air or hyperoxia groups. Two-way ANOVA showed an effect of hyperoxia and genotype and a two-way interaction. Significant differences between air-breathing and hyperoxia-exposed animals are indicated by *, $p < 0.05$. Significant differences between hyperoxia-exposed WT and AhRd mice are indicated by †, $p < 0.05$.