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β -Naphthoflavone treatment attenuates neonatal hyperoxic lung injury in wild type *Cyp1a2*-knockout mice

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Abstract

Exposure to supraphysiological concentrations of oxygen (hyperoxia) leads to bronchopulmonary dysplasia (BPD), one of the most common pulmonary morbidities in preterm neonates, which is more prevalent in males than females. Beta-naphthoflavone (BNF) is protective against hyperoxic lung injury in adult and neonatal wild type (WT) mice and in mice lacking *Cyp1a1* gene. In this investigation, we tested the hypothesis that BNF treatment will attenuate neonatal hyperoxic lung injury in WT and *Cyp1a2*^{-/-} mice, and elucidated the effect of sex-specific differences. Newborn WT or *Cyp1a2*^{-/-} mice were treated with BNF (10 mg/kg) or the vehicle corn oil (CO) i.p., from postnatal day (PND) 2 to 8 once every other day, while being maintained in room air or hyperoxia (85% O₂) for 14 days. Hyperoxia exposure lead to alveolar simplification and arrest in angiogenesis in WT as well as *Cyp1a2*^{-/-} mice. No significant differences were seen between WT and *Cyp1a2*^{-/-} mice. *Cyp1a2*^{-/-} female mice had better preservation of pulmonary angiogenesis at PND15 compared to similarly exposed males. BNF treatment attenuated lung injury and inflammation in both genotypes, and this was accompanied by a significant induction of hepatic and pulmonary CYP1A1 in WT but not in *Cyp1a2*^{-/-} mice. BNF treatment increased NADPH quinone oxidoreductase (NQO1) mRNA levels in *Cyp1a2*^{-/-} mouse livers compared to WT mice. These results suggest that BNF is protective in neonatal mice exposed to hyperoxia independent of CYP1A2 and this may entail the protective effect of phase II enzymes like NQO1.

Keywords

Hyperoxia; Neonate; Bronchopulmonary Dysplasia; CYP1A2; beta-naphthoflavone

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INTRODUCTION

Exposure to supraphysiological concentrations of oxygen (hyperoxia) leads to arrest in alveolarization and angiogenesis in the developing lung. These are the hallmarks of bronchopulmonary dysplasia (BPD), one of the most common pulmonary morbidities in preterm neonates (Bhandari, 2010; Jobe, 2011). The alveoli are fewer in number and larger in size, decreasing the gas-exchange area in the lung. Also, the dysmorphic pulmonary vasculature leads to the development of pulmonary hypertension and additional long-term morbidities (Baker and Abman, 2015; Stenmark and Abman, 2005). Unfortunately, the incidence of BPD remains high despite advances in neonatal intensive care and has long-term consequences on lung function. There is an urgent need for therapeutic strategies that ameliorate the adverse effects of hyperoxia on the developing lung to decrease the incidence of BPD.

Hyperoxia exposure leads to the generation of reactive oxygen species (ROS), which in turn cause cellular damage in the lung by oxidizing nucleic acids, proteins, and membrane lipids. Previous studies have established the role of the cytochrome P450 (CYP) 1A subfamily in modulating hyperoxic lung injury (Bhakta et al., 2008; Couroucli et al., 2002; Okamoto et al., 1993; Shertzer et al., 2004; Sindhu et al., 2000). In mouse and humans, the CYP1A subfamily comprises of two genes; namely, *CYP1A1* (*Cyp1a1* in mice) and *CYP1A2* (*Cyp1a2* in mice). The CYP1A subfamily of enzymes metabolizes numerous endogenous and exogenous chemicals and their induction is regulated by the aryl hydrocarbon receptor (AHR). The AHR gene battery consists of many phase I and phase II metabolizing enzymes including CYP1A1, CYP1A2, CYP1B1, NQO1 and GSTA1. CYP1A1 is expressed mainly in the extra-hepatic tissues (rodent and human lung, intestine, placenta and kidney), while CYP1A2 is expressed mainly in the rodent and human liver. In a model of acute hyperoxic lung injury in adult mice, both *Cyp1a1*^{-/-} and *Cyp1a2*^{-/-} mice display a phenotype of exaggerated lung injury, inflammation and oxidative stress, thus highlighting the protective role of these enzymes in adult mice (Lingappan et al., 2014; 2017; Wang et al., 2015). In adult mice, hepatic CYP1A2 plays a critical role in the attenuation against hyperoxic lung injury by decreasing lipid peroxidation and oxidative stress *in vivo* (Wang et al., 2015). In neonatal mice, we have shown that prenatal induction of the CYP1A subfamily with BNF attenuated lung injury and that mice lacking the *Cyp1a1* gene have greater arrest in alveolarization and angiogenesis when exposed to postnatal hyperoxia (Couroucli et al., 2011; Maturu et al., 2017). Exposure to postnatal hyperoxia for varying duration leads to a lung phenotype comprising of alveolar simplification similar to that observed in human neonates with BPD (Berger and Bhandari, 2014).

The expression of CYP1A2 is minimal in the fetal and neonatal liver, and the expression gradually increases beyond the second week of life (Hart et al., 2009). However, the enzyme is inducible in newborn mice (Couroucli et al., 2011). Prenatal administration of beta-naphthoflavone for three days on gestational days (E17–19) protected neonatal mice against lung injury, when subjected to hyperoxia postnatally. This was accompanied by induction in *Cyp1a2* gene expression both in the fetal and neonatal liver (Couroucli et al., 2011). The role of CYP1A2 in modulating hyperoxic lung injury in the newborn period has not been investigated. Sex-specific differences have been observed in the incidence of BPD in

premature neonates with male infants showing a greater predilection for the development of this disease in multiple clinical studies (Shim et al., 2017). In adult and neonatal mice exposed to hyperoxia, similar pattern was observed with increased lung injury observed in male mice (Lingappan et al., 2013; 2016). Differential expression of CYP1A1 and CYP1A2 was also observed (Lingappan et al., 2015). We hypothesized that in the absence of the *Cyp1a2* gene BNF will not have protective effect in the setting of neonatal hyperoxia exposure. We also tested the effect of sex as a biological variable on the outcome measures related to lung development and angiogenesis in this experimental model.

MATERIALS AND METHODS

Animals

This study was conducted in accordance with the federal guidelines for the humane care and use of laboratory animals, and was approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine. Breeding pairs of WT mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Breeding pairs of *Cyp1a2*^{-/-} mice of mixed background (C57BL/6J/Sv129) were obtained from Dr. Frank J. Gonzalez (National Cancer Institute, Bethesda, MD). The mice were backcrossed with C57BL/6J mice for 12 generations to generate theoretically a >99.2% C57BL/6J background. The dams were fed standard mice food (Purina Rodent Lab Chow 5001 from Purina Mills, Inc., Richmond, IN) and water *ad libitum*. Animals were maintained in 12-h day/night cycles.

Hyperoxia exposure and tissue collection

Newborn pups and dams of either genotype were placed in plexi-glass chambers within 12 hours of birth and were exposed to either 21% O₂ (room air) or 85% O₂ from PND 1–14. This period extends through the saccular and alveolar stages of lung development in the mouse. This model has been validated to produce a phenotype similar to the human BPD (Hilgendorff et al., 2013). Oxygen was delivered through a humidified circuit at a flow rate of 5 L/min through a blender to was monitored continuously by achieve an inspired oxygen concentration of 85%. FiO₂ means of in-line analyzers at the out port of the chambers. The dams were rotated between room air and hyperoxia-exposed litters every 24 h to prevent oxygen toxicity in the dams. Mouse pups were treated with BNF (10 mg/kg) dissolved in corn oil (CO) or CO, intraperitoneally (i.p.) once every other day with, from postnatal days (PND) 2 to 8. The mice were sacrificed on PND 15. The animals were anesthetized with sodium pentobarbital (200 mg/kg i.p.) and euthanized by exsanguination while under deep pentobarbital anesthesia. Six animals from each group (three males and three females) were used for lung histopathology. Lung and liver tissues were harvested for mRNA and protein isolation. Lung weight and body weight were also measured on PND15.

Analysis of Lung Morphometry

Lungs were inflated through an intratracheal catheter with buffered zinc formalin (10%) and fixed in with the same solution at a constant pressure of 25 cm H₂O for at least 10 min. Samples were left in solution for 24 h in 10% zinc formalin, and then transferred to 70% ethanol for long-term storage, following which they were embedded in paraffin for subsequent histological analyses. Lungs were sectioned at 4 μm thickness on a rotary

microtome and were stained with hematoxylin and eosin (H&E). Alveolar development was evaluated at PND15 (n=6/group) by radial alveolar counts (RAC) (Cooney and Thurlbeck, 1982) and mean linear intercept (MLI) (McGowan et al., 2000) as described before (Nicola et al., 2009). Fifteen randomly chosen areas were photographed (200x magnification). Fields containing large airways and vessels were not included. Analysis of each section was carried out in a blinded fashion

Immunohistochemistry and quantitation for assessment of angiogenesis and inflammatory cell infiltration in the lungs

Pulmonary vascular density was measured in lung sections stained for Von Willebrand factor (VWF) with anti-Von Willebrand Factor polyclonal antibody (Abcam-Cat# ab6994) at 1:500 dilution. Ten random non-overlapping fields were assessed at 200x magnification for each animal (n=6/group; 3 males and 3 females). Neutrophils were immunostained with rat anti-mouse ly-6b.2 antibody (AbD Serotec; catalog number: MCA771G) at 1:200 dilution and macrophages using the anti-F4/80 antibody (1:500 dilution, Bio Rad laboratories; catalog number: MCA497GA). Quantitation of neutrophils and macrophages was performed by averaging the counts from at least 20 random high power fields.

Liver and Lung tissue western blotting

The snap frozen lung samples were weighed and added to 1 ml of lysis buffer (PBS+ cocktail of protease inhibitor). The tissues were ground and the samples were incubated on a rocker at 4°C for 15 minutes followed by centrifugation at 10,000 G for 15 minutes. The supernatants were removed and protein concentrations were determined using the BCA (bicinchoninic acid) method. Liver and lung whole protein homogenates (20 µg) prepared from individual animals (n=3/group) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels. The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by Western blotting using monoclonal antibodies to detect CYP1A1 (1:1500 dilution), which cross-reacts with CYP1A2 (Couroucli *et al.*, 2002; Jiang *et al.*, 2004; Moorthy *et al.*, 2000) and NQO1 mouse monoclonal antibody (Santa Cruz-Cat#393736; 1:1000 dilution). β-actin was used as the loading control. This was followed by electrochemical detection of bands. Band intensities were quantified using Image Lab™ 6.0 Software (Bio Rad laboratories) (Taylor et al., 2013).

CYP1A1 (EROD) and CYP1A2 (MROD) assays

Lung and liver samples at the time of dissection were frozen immediately with liquid nitrogen and maintained at a temperature of – 80°C. Homogenized lung and liver tissues were mixed with Tris-sucrose buffer (0.25M sucrose, 80 mM Tris, 5 mM MgCl₂, 0.25 mM KCl, 1 mM EDTA) pH 7.4 and centrifuged at 12 000 rpm for 10 min. The supernatant was collected and after estimation of protein concentration was subjected to EROD and MROD assays (n=3/group). Ethoxyresorufin *O*-deethylase (EROD) (CYP1A1) activities in lung and liver and methoxyresorufin *O*-demethylase (MROD) (CYP1A2) activities in liver tissues were assayed as described previously (Moorthy et al., 1997).

Quantitative Real time PCR assays

RNA was isolated from snap frozen liver and lung samples using Direct-Zol RNA mini prep kit (Zymo research, Cat#R2052) with a DNase step, according to the instructions of the manufacturer. For quantitative real-time PCR, 1 µg of total RNA from livers and lungs with different treatment conditions was reverse-transcribed in 20 µL of using iScript Reverse Transcription super mix (Biorad-Cat#170-8841) according to manufacturer's instructions. The cDNA (2µl) and the real-time PCR primers for CYP1A1 (Forward 5'-GGTTAACCATGACCGGGAAC-3' Reverse 5'-TGCCCAAACCAAAGAGAGTGA-3') and NQO1 (Forward 5'-GGAAGCTGCAGACCTGGTGA-3' Reverse 5'-CCTTTCAGAATGGCTGGCA-3') were used in final 20 µL qPCR reaction with a SYBR-green master mix (Qiagen-Cat#2014143). Real-time qPCR was performed in an ABI-Prism7700 sequence detection system (n=3/group). PCR conditions were with denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 minute, with 40 PCR cycles. Data was analyzed by Ct method; the expression of the target genes such as CYP1A1 and NQO1 were normalized to β -Actin as an endogenous control.

Statistical analysis

Based on preliminary results of hyperoxia-induced alveolar simplification in WT neonatal mice, it was estimated that 5 animals per group would provide 80% power at a significance level of 0.05 for the effect size of 5 µm in mean linear intercept (mean difference) between the two genotypes (WT and *Cyp1a2*^{-/-}). Data are expressed as means $\pm$ SEM. 3-way analyses of variance (ANOVA) (effect of genotype and BNF-treatment or sex and hyperoxia exposure), followed by modified t-tests, were used to assess significant differences arising from exposure to hyperoxia and room air, WT and *Cyp1a2*^{-/-} mice, male and female mice and BNF and corn-oil treated mice. *P*-values of < 0.05 were considered significant.

RESULTS

Effect of hyperoxia on Body weight (BW) and Lung weight (LW)

Cyp1a2^{-/-} mice weighed less than WT mice on PND15 in room air. Chronic hyperoxia exposure led to a significant weight loss in WT mice and BNF treatment prevented this. In *Cyp1a2*^{-/-} mice, BNF treatment led to an increase in body weight both in room air and under hyperoxic conditions compared to unexposed controls (Figure 1A). Lung weights did not show a statistically significant increase in any group after hyperoxia exposure; however BNF treatment decreased lung weight under hyperoxic conditions in WT mice compared to unexposed controls (Figure 1B).

Neonatal WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia had increased alveolar simplification, which was ameliorated by BNF

WT and *Cyp1a2*^{-/-} newborn mice were exposed to room air or 85% O₂ from PND1–14 and lung morphometry was assessed to quantitate the effect on lung development. Representative lung fields from room air and hyperoxia exposed WT (Figure 2A–D) and *Cyp1a2*^{-/-} mice (Figure 2E–H) are shown at 20x magnification. Exposure to hyperoxia caused dramatic alveolar simplification as seen in the lung sections with fewer and larger alveoli in both WT

and *Cyp1a2*^{-/-} mice. Alveolar size was quantified using mean linear intercept (MLI) and radial alveolar count (RAC) using established methods. These results are shown in Figure 3A–F. Hyperoxia significantly increased MLI and decreased RAC in hyperoxia-exposed mice of either genotype. Analysis by 3-way ANOVA showed that for MLI, effect of hyperoxia treatment was significant; while the effects of sex and genotype were not significant. In mice with BNF administration, radial alveolar count was decreased to a greater extent in *Cyp1a2*^{-/-} males compared to WT males. There were no sex-specific differences noted. When sexes were combined, BNF treatment attenuated the adverse impact of hyperoxia on alveolarization and preserved MLI in both WT and *Cyp1a2*^{-/-} mice and preserved RAC in WT mice (Figure 3C and F).

Postnatal hyperoxia exposure leads to arrest in angiogenesis in both WT and *Cyp1a2*^{-/-} mice and is recued by BNF treatment

To assess differences in angiogenesis between WT and *Cyp1a2*^{-/-} neonatal mice with or without BNF treatment, we quantified vessel number using ant-vWF antibodies. Representative immunostained lung sections are shown in figures 4A–H. Hyperoxia significantly decreased vessel development in the lung in both WT and *Cyp1a2*^{-/-} mice of either sex (Figure 4I); however, *Cyp1a2*^{-/-} males had a greater arrest in angiogenesis compared to *Cyp1a2*^{-/-} females, upon exposure to hyperoxia. Treatment with BNF ameliorated this process to a greater extent in WT mice compared to *Cyp1a2*^{-/-} mice (Figure 4K). Among WT mice, males displayed no significant arrest in angiogenesis with BNF treatment, while this was still observed in *Cyp1a2*^{-/-} males (Figure 4J).

Effect of hyperoxia on pulmonary macrophage and neutrophil infiltration

Exposure to hyperoxia caused a significant increase in macrophage infiltration in the lungs of WT mice of either sex (Figure 5A), this was significantly decreased by BNF treatment compared to corn-oil treated controls (Figure 5C). *Cyp1a2*^{-/-} mice did not show increased macrophage infiltration in the lungs upon exposure to hyperoxia. Among BNF treated mice, no difference was seen due to hyperoxia treatment or between WT mice and *Cyp1a2*^{-/-} mice (Figure 5B). Representative lung sections from room-air or hyperoxia exposed WT mice (with corn-oil or BNF treatment) stained for macrophages are shown in Figures 5D. Similarly, neutrophils were increased in the lungs of corn-oil treated WT mice exposed to hyperoxia compared to room air controls of either sex and no significant increase was seen in *Cyp1a2*^{-/-} mice (Figure 5E). Among BNF treated mice, WT males had decreased pulmonary neutrophils after hyperoxia treatment (Figure 5F). When both sexes were combined, analysis showed that BNF treatment significantly decreased neutrophil recruitment in the lung of hyperoxia-exposed WT mice compared to room-air controls (Figure 5G). Representative lung sections immunostained for neutrophils from WT mice are shown in Figure 5H.

Pulmonary and hepatic expression of *Cyp1a1* and *Cyp1a2* after hyperoxia exposure in WT and *Cyp1a2*^{-/-} mice

Upon exposure to hyperoxia, there was a significant induction of *Cyp1a1* in WT mice administered corn-oil (Figure 6A). A similar induction was noted in BNF treated WT mice exposed to hyperoxia and the increase was greater compared to corn-oil treated controls. In

Cyp1a2^{-/-} mice, there was no induction in *Cyp1a1* expression under hyperoxia and no increase was seen with BNF treatment. Thus, genotype had a significant effect on pulmonary *Cyp1a1* expression and absence of the *Cyp1a2* gene abrogated the induction in *Cyp1a1* mRNA expression in the lung under hyperoxic conditions in *Cyp1a2*^{-/-} mice. Western blot analysis for CYP1A1 protein expression (Figure 6B) showed an induction in WT mice treated with BNF under room air. The significant induction in gene expression under hyperoxia was not observed in the expression of the corresponding protein. This difference between mRNA and protein levels may be attributed to alteration in post-transcriptional regulation or due to increased protein catabolism, including the ubiquitin-proteasome system as shown previously (Chambellan et al., 2006). Similar to mRNA levels, *Cyp1a2*^{-/-} mice showed no induction of CYP1A1 either under hyperoxic conditions or with BNF treatment. In the liver, BNF administration lead to a significant increase in CYP1A1 expression in normoxic mice compared to corn-oil treated controls. Hyperoxia exposure significantly decreased hepatic *Cyp1a1* expression in BNF treated WT mice. *Cyp1a2*^{-/-} mice did not show a significant increase in hepatic *Cyp1a1* expression despite BNF treatment. This was similar to the absence of induction in *Cyp1a1* gene expression noted in the lung in *Cyp1a2*^{-/-} mice. Liver western blot (Figure 6D) showed induction of CYP1A1 in BNF treated WT mice as shown in Figure 6C. A CYP1A1 protein band was not detected in any of the other experimental groups neither in WT mice nor in *Cyp1a2*^{-/-} mice.

CYP1A1 (EROD) and CYP1A2 (MROD) activity in in WT and *Cyp1a2*^{-/-} mice upon hyperoxia exposure

Liver MROD assay, which measures CYP1A2 activity, showed a significant increase in BNF treated WT mice under room air conditions, and a significant decrease with hyperoxia exposure. As expected, MROD activity in *Cyp1a2*^{-/-} mice was very low (Figure 7A). Liver EROD assay measures the CYP1A1 activity. There was a significant induction in EROD activity with BNF administration in WT mice, which was decreased upon hyperoxia exposure. Similar to mRNA levels, there was no induction in EROD (CYP1A1) activity in *Cyp1a2*^{-/-} mice either with hyperoxia exposure or BNF administration (Figure 7B). Lung EROD activity increased with BNF administration in WT mice and decreased following hyperoxia exposure (Figure 7C). This was similar to the trend observed with pulmonary CYP1A1 protein levels as shown in Figure 6B. *Cyp1a2*^{-/-} mice did not show any induction in lung EROD under hyperoxia or with BNF treatment.

Pulmonary and hepatic expression Nqo1 after hyperoxia exposure in WT and *Cyp1a2*^{-/-} mice

NAD(P)H:quinone oxidoreductase 1 (*NQO1*) is a major antioxidant/phase II gene in the lung. We measured *Nqo1* mRNA and protein expression in WT and *Cyp1a2*^{-/-} mice under the different experimental conditions. In WT mice, changes in hepatic *Nqo1* mRNA expression were not statistically significant. Interestingly, corn-oil treated *Cyp1a2*^{-/-} mice showed increased hepatic *Nqo1* mRNA expression under hyperoxic conditions. Compared to corn-oil treated controls, BNF treatment led to increase in *Nqo1* mRNA expression in *Cyp1a2*^{-/-} mice. NQO1 protein levels increase with BNF treatment in the livers of WT mice in room air with a decrease following hyperoxia exposure (Figure 8A). *Cyp1a2*^{-/-} mice also showed a similar pattern in liver NQO1 protein expression (Figure 8B).

In the lung, BNF treatment did not significantly increase *Nqo1* mRNA expression in WT mice compared to corn-oil treated controls. Hyperoxia exposure induced expression in both corn-oil and BNF treated WT and *Cyp1a2*^{-/-} mice. BNF treatment significantly increased *Nqo1* mRNA expression in *Cyp1a2*^{-/-} mice both in room air and hyperoxic conditions (Figure 8C). This induction was significantly different in *Cyp1a2*^{-/-} mice compared to similarly treated WT mice in room air. Lung NQO1 protein levels were increased with hyperoxia exposure in WT mice both in corn-oil and BNF treated mice and this was greater in BNF-treated mice compared to corn-oil treated controls (Figure 8D). Corn-oil treated *Cyp1a2*^{-/-} mice showed increased NQO1 protein expression in the lung after hyperoxia exposure.

DISCUSSION

The role of the CYP1A subfamily of enzymes in hyperoxic lung injury have been reported in many previous publications (Lingappan et al., 2014; Okamoto et al., 1993; Sinha et al., 2005; Wang et al., 2015). Induction of CYP1A is protective against, while inhibition increases hyperoxic lung injury in adult mice (Moorthy et al., 2000). In neonatal mice, we have shown that prenatal administration of CYP1A inducer (BNF) attenuates lung injury and preserves lung development (Couroucli et al., 2011). *Cyp1a1*^{-/-} mice have greater alveolar simplification and impairment in angiogenesis compared to WT mice following postnatal hyperoxia exposure (Maturu et al., 2017). *Cyp1a1*^{-/-} mice displayed a greater induction of hepatic CYP1A2 and pulmonary NQO1 enzymes and the protective effect of BNF was speculated to be mediated through these mechanisms.

The primary objective of this investigation was to determine the role of hepatic CYP1A2 in neonatal hyperoxic lung injury using mice lacking the gene for CYP1A2. CYP1A2 is expressed in the liver, and is absent in the lung. *Cyp1a2*^{-/-} adult mice exposed to hyperoxia display increased lung injury, pulmonary edema and inflammation compared to similarly exposed WT mice (Wang et al., 2015). This led us to speculate that liver CYP1A2 may be imparting a protective role in the setting of hyperoxic lung injury. However, in neonatal mice, absence of *Cyp1a2* did not increase susceptibility to hyperoxic lung injury and did not attenuate the protective effect of BNF. Lung alveolarization as measured by morphometric indices such as MLI and RAC, though adversely impacted by hyperoxia, were not different between WT and *Cyp1a2*^{-/-} mice. The interesting finding in this manuscript is the sharp contrast between the adult (previously published; Wang et al., 2015) and neonatal lung phenotype in *Cyp1a2*^{-/-} mice in the setting of hyperoxic lung injury and in the expression of *Cyp1a1* in *Cyp1a2*^{-/-} mice. In the *Cyp1a2*^{-/-} neonatal mice, *Cyp1a1* expression was low at baseline and no induction was seen with hyperoxia or with BNF treatment. In contrast, in adult *Cyp1a2*^{-/-} mice, there was a marked increase in pulmonary *Cyp1a1* expression. Also, the increased expression of NQO1 in *Cyp1a2*^{-/-} neonatal mice after BNF treatment has not been reported before and this may attenuate the effect of hyperoxia on the developing lung in these animals. BNF administration ameliorated the adverse impact of hyperoxia in both WT and *Cyp1a2*^{-/-} mice by improving alveolarization, angiogenesis, *Nqo1* expression and decreasing inflammation.

Since, the *Cyp1a2*^{-/-} mice weighed less than WT mice at PND15, we did not use lung weight/body weight ratios as a marker of lung injury in the present manuscript. Instead we reported body weight and lung weight separately. BNF had a strong effect in preventing weight loss in hyperoxia exposed *Cyp1a2*^{-/-} neonatal mouse pups. Several differences can be highlighted based on the findings in the current study and previously published results on the role of CYP1A2 in adult and neonatal lungs in the setting of hyperoxia exposure. *Cyp1a2*^{-/-} adult mice showed increased expression of *Cyp1a1* in the liver and lung (Wang et al., 2015). Endogenous AHR ligands, which may be metabolized by CYP1A2, could accumulate in *Cyp1a2*^{-/-} adult mice, thereby leading to CYP1A1 induction even in room air. This was not observed in neonatal mice and CYP1A1 was not inducible in these mice even after BNF administration. This suggests that absence of CYP1A2 leads to CYP1A1 induction in adult mice but not in neonatal mice. *Cyp1a2*^{-/-} adult mice showed increased pulmonary neutrophil infiltration upon exposure to hyperoxia. In the neonates, in the present study, there was no increase in pulmonary or macrophage recruitment in the lung in *Cyp1a2*^{-/-} neonatal mice, while WT mice showed increase in both. Developmental differences in key biological pathways modulating hyperoxic lung injury have been reported by other investigators (Wright et al., 2009); (Yang et al., 2004); (Yang et al., 2000).

Cyp1a2 is not expressed in the neonatal liver in the first 2 weeks, with a slow increase in expression noted thereafter (Selwyn et al., 2015). In a study by Hart *et al*, describing the ontogeny of different cytochrome P450 genes in the maturing mouse liver, *Cyp1a2* expression gradually increased after birth till PND20 and remained constant until PND45 (Hart et al., 2009). Similar findings were reported in neonatal rats with immunodetectable CYP1A2 at PND3 (at approximately 25% of adult levels) with gradual increase reaching 50% of adult levels by PND42 and adult levels by 6 to 9 weeks of life (Elbarbry et al., 2007). In human livers, a similar delay in the ontogeny of CYP1A2 expression has been reported (Sonnier and Cresteil, 1998). CYP1A2 is not detectable in the immediate perinatal period with levels slowly increasing beyond infancy. However, CYP1A2 is inducible in the neonatal murine liver with prenatal (Couroucli et al., 2011) or neonatal administration of BNF (CYP1A inducer) (Maturu et al., 2017), resulting in increased expression of mRNA and protein levels of CYP1A2 and attenuated hyperoxic lung injury. Based on these data and the increased lung injury noted in *Cyp1a2*^{-/-} adult mice led to the hypothesis that absence of the inducible CYP1A2 in *Cyp1a2*^{-/-} neonatal mice would partially decrease the beneficial effect of BNF in the model of postnatal hyperoxia exposure. However, results from this study show that BNF has beneficial effects in *Cyp1a2*^{-/-} neonatal mice to the same extent as WT mice. This shows that BNF treatment attenuates hyperoxic lung injury independent of CYP1A2 induction probably through mechanisms including upregulation of other antioxidant genes such as *Nqo1* or due its anti-inflammatory and pro-angiogenic effect.

Despite the well-established sex-specific differences in the incidence of BPD and impaired lung function in males, the molecular mechanism(s) behind this are not completely understood (Stevenson et al., 2000). There are sex-related differences in the constitutive expression of CYP1A *in vivo* (Asaoka et al., 2010; Eber and Zach, 2001; Kojima et al., 2010; 2008; Rasmussen et al., 2011) and differential modulation of CYP1A by testosterone and estradiol *in vitro* (Lee et al., 1998; Monostory et al., 2009; Spink et al., 2003). We have

shown that exposure to hyperoxia leads to a greater induction in the expression and activity of CYP1A in female WT adult mice in a model of acute hyperoxic lung injury (Lingappan et al., 2013). BNF treated *Cyp1a2*^{-/-} male mice showed greater reduction in radial alveolar count (a marker of alveolarization; Figure 3E) and angiogenesis (Figure 4J) than WT male mice. *Cyp1a2*^{-/-} female mice had better preservation of pulmonary angiogenesis at PND15 compared to similarly exposed males (Figure 4I).

NF-E2-related factor-2 (Nrf2) activates the antioxidant response elements (ARE) pathway which comprises of many genes which regulate the cellular response to oxidative stress (Cho et al., 2002; Cho and Kleeberger, 2015). One of these genes is NAD(P)H:quinone oxidoreductase 1 (*NQO1*), which plays a role as a major antioxidant/phase II gene in the lung. By catalyzing two-electron reduction of a variety of quinone compounds, it prevents generation of free radicals and protects cells from oxidative damage. Single nucleotide polymorphisms in the region of the *NQO1* gene were found to be associated with increased risk for the development of BPD in human patients (Sampath et al., 2015). Induction of *NQO1* in the lung by hyperoxia exposure has been reported in both adult and neonatal lungs (Cho et al., 2002; Maturu et al., 2017). Mice deficient in *Nqo1* were more susceptible to hyperoxic lung injury (Das et al., 2006). Based on the previously established evidence, we measured *Nqo1* expression in WT and *Cyp1a2*^{-/-} mice after BNF expression if this could be in part responsible for the protective effect of BNF in this model. In this study, we show significant induction in pulmonary *Nqo1* expression by hyperoxia in both WT and *Cyp1a2*^{-/-} mice. The response to BNF treatment was greater in *Cyp1a2*^{-/-} mice. This may have provided some protection against oxidative stress in the setting of postnatal hyperoxia exposure.

In conclusion, absence of *Cyp1a2* gene in the neonatal mouse does not increase hyperoxia-mediated arrest in alveolarization and pulmonary angiogenesis. Treatment with BNF imparts protection to both WT and *Cyp1a2*^{-/-} mice with preservation of lung alveolar and vascular development and attenuation of inflammation in WT mice. Several differences between the neonatal and adult phenotype based on previously published findings were also highlighted. *Cyp1a2*^{-/-} mice showed a robust induction in *Nqo1* expression with hyperoxia and BNF treatment, which could in part play a protective role in this model of postnatal hyperoxia exposure. Further studies are needed to understand the mechanistic role of CYP1A2 in neonatal lung injury, as well as the protective effects of BNF. These studies could lead to additional translational studies that could benefit preterm infants undergoing supplemental oxygen therapy.

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Highlights

- BNF administration ameliorates lung injury in both wild type and *Cyp1a2*^{-/-} mice.
- *Cyp1a2*^{-/-} mice show increased expression of *Nqo1* mRNA after BNF treatment.
- NQO1 can contribute to the protective effects of BNF

Figure 1. Effect of hyperoxia on Body weight (BW) and Lung weight (LW)

Figure 1A shows the effect of hyperoxia exposure (PND 1–14; 85% FiO₂) on body weights of WT and *Cyp1a2*^{-/-} mice treated with corn-oil or BNF. **1B:** Effect of hyperoxia exposure (PND 1–14; 85% FiO₂) on body weights of WT and *Cyp1a2*^{-/-} mice treated with corn-oil or BNF. Values are means ± SEM from at least 5–10 individual animals. Significant differences between room air and hyperoxia exposed mice are indicated by *, p<0.05. Differences between WT and *Cyp1a2*^{-/-} mice are indicated by δ , p <0.05 and those between corn-oil and BNF treated are indicated by ##, p <0.01 and ###, p<0.001.

Figure 2. Effect of hyperoxia on lung histopathology

Representative hematoxylin and eosin stained images from the lungs of WT and *Cyp1a2*^{-/-} mice (n=6 mice per group) WT and *Cyp1a2*^{-/-} mice were exposed to hyperoxia (PND 1–14; 85% FiO₂) and treated with corn-oil or BNF. Hyperoxia exposed WT and *Cyp1a2*^{-/-} mice show more alveolar simplification compared to mice in room-air conditions. Treatment with BNG ameliorates the adverse effects of hyperoxia on lung alveolarization.

Figure 3. Effect of hyperoxia on lung morphometry

To quantitate the effects of hyperoxia exposure on alveolarization, mean linear intercept (Figures 3A–C) and radial alveolar count (Figures 3D–F) was measured in WT and *Cyp1a2*^{-/-} mice. Figure 3A: Mean linear intercept in male and female mice (WT and *Cyp1a2*^{-/-}) in the corn-oil group. Figure 3B: Mean linear intercept in male and female mice (WT and *Cyp1a2*^{-/-}) that received treatment with BNF. Figure 3C: Effect of BNF treatment on MLI under hyperoxia and room air conditions compared to corn-oil treated controls. Figure 3D: Radial alveolar count (RAC) in male and female mice (WT and *Cyp1a2*^{-/-}) in the corn-oil group. Figure 3E: RAC in male and female mice (WT and *Cyp1a2*^{-/-}) that received treatment with BNF. Figure 3F: Effect of BNF treatment on RAC under hyperoxia and room air conditions compared to corn-oil treated controls. Values are means $\pm$ SEM from at least 3 individual animals of each sex and genotype. Significant differences between room air and hyperoxia exposed mice are indicated by *, $p < 0.05$ and ***, $p < 0.001$. Differences between WT and *Cyp1a2*^{-/-} mice are indicated by δ , $p < 0.05$ and those between corn-oil and BNF treated are indicated by ###, $p < 0.001$.

Figure 4. Effect of hyperoxia on lung vessel development

To assess differences in angiogenesis among WT and *Cyp1a2*^{-/-} neonatal mice with or without BNF treatment, we quantified vessel number using ant-vWF antibodies with immunohistochemistry on PND15 after the completion of hyperoxia exposure.

Representative lung sections from all the experimental groups are shown in figure panels A–H (4A–D in WT and 4E–H in *Cyp1a2*^{-/-} mice). Arrows point to brown-staining vessels.

Figures 4I–K: Quantitative analyses showing number of vessels per high-power field in lungs of WT and *Cyp1a2*^{-/-} neonatal mice. Figure 4I: Vessel number/high power field (hpf) in male and female mice (WT and *Cyp1a2*^{-/-}) in the corn-oil group. Figure 4J: Vessel number/hpf in male and female mice (WT and *Cyp1a2*^{-/-}) that received treatment with BNF. Figure 4K: Effect of BNF treatment on vessel number/hpf under hyperoxia and room air conditions compared to corn-oil treated controls. Values are means ± SEM from at least 3 individual animals of each sex and genotype. Significant differences between room air and hyperoxia exposed mice are indicated by *, p<0.05, **, p<0.01 and ***, p<0.001. Differences between WT and *Cyp1a2*^{-/-} mice are indicated by δ, p<0.05 and those between corn-oil and BNF treated are indicated by #, p<0.05 and ###, p<0.001.

Figure 5. Effect of hyperoxia on pulmonary neutrophil and macrophage infiltration

Representative lung sections (40x magnification) immunostained for macrophages (Figure 5D) and neutrophils (Figure 5H) in lungs of WT and *Cyp1a2*^{-/-} neonatal mice. Quantitation of macrophages (Figure 5A–C) and neutrophils (5E–G) in the different experimental groups is also shown. Figure 5A: Macrophages/high power field (hpf) in male and female mice (WT and *Cyp1a2*^{-/-}) in the corn-oil group. Figure 5B: Macrophages/hpf in male and female mice (WT and *Cyp1a2*^{-/-}) that received treatment with BNF. Figure 5C: Effect of BNF treatment on macrophages/hpf under hyperoxia and room air conditions compared to corn-oil treated controls. Figure 5E: Neutrophils/high power field (hpf) in male and female mice (WT and *Cyp1a2*^{-/-}) in the corn-oil group. Figure 5F: Neutrophils/hpf in male and female mice (WT and *Cyp1a2*^{-/-}) that received treatment with BNF. Figure 5G: Effect of BNF treatment on neutrophils/hpf under hyperoxia and room air conditions compared to corn-oil treated controls. Values are means $\pm$ SEM from at least 3 individual animals of each sex and genotype. Significant differences between room air and hyperoxia exposed mice are indicated by *, $p < 0.05$. Differences between corn-oil and BNF treated are indicated by #, $p < 0.05$ and ###, $p < 0.001$.

Figure 6. Effect of hyperoxia on pulmonary and hepatic Cyp1a1 mRNA and CYP1A1 protein expression in WT and *Cyp1a2*^{-/-} mice

Figure 6A: Pulmonary *Cyp1a1* mRNA expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. Figure 6B: Representative western blot image and densitometric analysis of lung CYP1A1 protein expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. The positive control (labeled “PC”) was 0.5 µg of liver microsomes from mice treated with 3-MC. Figure 6C: Hepatic *Cyp1a1* mRNA expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. Figure 6D: Representative western blot image of liver CYP1A1 protein expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. “PC” refers to positive control as described above. Values are means ± SEM from at least 3 individual animals of each genotype. Significant differences between room air and hyperoxia exposed mice are indicated by **, p<0.01 and ***, p<0.001. Differences between corn-oil and BNF treated are indicated by ###, p<0.001. Differences between WT and *Cyp1a2*^{-/-} mice are indicated by \$\$\$, p<0.001.

Figure 7. Effect of hyperoxia on pulmonary and hepatic EROD (CYP1A1) and MROD (CYP1A2) activity in WT and *Cyp1a2*^{-/-} mice

Liver MROD (Figure 7A) and EROD (Figure 7B) and Lung EROD (Figure 7C) activity in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. Values are means $\pm$ SEM from at least 3 individual animals of each genotype. Significant differences between room air and hyperoxia exposed mice are indicated by *, $p < 0.05$ and ***, $p < 0.001$. Differences between corn-oil and BNF treated are indicated by #, $p < 0.05$ and ###, $p < 0.001$. Differences between WT and *Cyp1a2*^{-/-} mice are indicated by $\delta\delta\delta$, $p < 0.001$.

Figure 8. Effect of hyperoxia on pulmonary and hepatic *Nqo1* mRNA and NQO1 protein expression in WT and *Cyp1a2*^{-/-} mice

Figure 8A: Hepatic *Nqo1* mRNA expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. Figure 8B: Representative western blot image and densitometric analysis of liver NQO1 protein expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. Figure 8C: Pulmonary *Nqo1* mRNA expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. Figure 8D: Representative western blot image of lung NQO1 protein expression in WT and *Cyp1a2*^{-/-} mice exposed to hyperoxia or room air that received corn-oil or BNF treatment. Values are means $\pm$ SEM from at least 3 individual animals of each genotype. Significant differences between room air and hyperoxia exposed mice are indicated by *, $p < 0.05$, and **, $p < 0.01$. Differences between corn-oil and BNF treated are indicated by #, $p < 0.05$, ##, $p < 0.01$ and ###, $p < 0.001$. Differences between WT and *Cyp1a2*^{-/-} mice are indicated by δ , $p < 0.05$.