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Increased susceptibility to hyperoxic lung injury and alveolar simplification in newborn rats by prenatal administration of benzo[a]pyrene

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Abstract

Maternal smoking is one of the risk factors for preterm birth and for the development of bronchopulmonary dysplasia (BPD). In this study, we tested the hypothesis that prenatal exposure of rats to benzo[a]pyrene (BP), a component of cigarette smoke, will result in increased susceptibility of newborns to oxygen-mediated lung injury and alveolar simplification, and that cytochrome P450 (CYP)1A and 1B1 enzymes and oxidative stress mechanistically contribute to this phenomenon. Timed pregnant Fisher 344 rats were administered BP (25 mg/Kg) or the vehicle corn oil (CO) on gestational days 18, 19 and 20, and newborn were either maintained in room air or exposed to hyperoxia (85% O₂) for 7 or 14 days. Hyperoxic newborn rats prenatally exposed to the vehicle CO showed lung injury and alveolar simplification, and inflammation, and these effects were potentiated in rats that were prenatally exposed to BP. Prenatal exposure to BP, followed by hyperoxia, also resulted in significant modulation of hepatic and pulmonary cytochrome P450 (CYP)1A and 1B1 enzymes at PND 7-14. These rats displayed significant oxidative stress in lungs at postnatal day (PND) 14, as evidenced by increased levels of the F₂-isoprostane 8-iso-PGF₂α. Furthermore, these animals showed BP-derived DNA adducts and oxidative DNA adducts in the lung. In conclusion, our results show increased susceptibility of newborns to oxygen-mediated lung injury and alveolar simplification following maternal exposure

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to BP, and our results suggest that modulation of CYP1A/1B1 enzymes, increases in oxidative stress, and BP-DNA adducts contributed to this phenomenon.

Keywords

Maternal benzo[a]pyrene; hyperoxic lung injury; newborn rat; cytochrome P4501A1/1A2/1B1; alveolar simplification; oxidative stress

1. Introduction

Maternal smoking is one of the risk factors for preterm birth and intra-uterine growth retardation (Andres and Day, 2000; Delpisheh et al., 2006; Huuskonen et al., 2008; Nieuwenhuijsen et al., 2013; Teune et al., 2012) resulting in serious health problems for the child during the neonatal period, which could in turn lead to chronic lifelong disorders such as cardiovascular disability, cerebral palsy, mental retardation, and even death (Barker et al., 1995; Horta et al., 1997; Jules et al., 2012). Intrauterine smoke exposure could also influence the development of bronchopulmonary dysplasia (BPD) (Antonucci et al., 2004; Singh et al., 2013). Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BP) are present in significant quantities in cigarette smoke. Maternal diet is also the main source of *in utero* exposure to contaminants such as PAHs (Phillips, 1999; Suzuki and Yoshinaga, 2007). PAHs cross the placental barrier, and therefore compromise fetal development (Autrup and Vestergaard, 1996; Sanyal et al., 2007).

BP is known human carcinogen, mutagen, and endocrine disruptor, and has been widely used as a marker for exposure of total carcinogenic PAH (Agency for Toxic Substances and Disease, 1995). Oral exposure to BP is known to induce developmental and reproductive toxicity in experimental studies in animals, including fetal growth (Duarte-Salles et al., 2013). Recent epidemiological studies suggest an association between dietary BP intake and lower birth weight in children (Duarte-Salles et al., 2013; Duarte-Salles et al., 2010). PAHs, by themselves are inert, but they induce enzymes such as cytochrome P450 (CYP)1A1, 1A2, and 1B1, which are involved in the activation of BP to reactive metabolites that in turn bind to protein and DNA, leading to carcinogenesis (Guengerich, 1988; Smerdova et al., 2013; Xue and Warshawsky, 2005). Maternal exposure to cigarette smoke induces CYP1A1 in placenta as well as fetus, resulting in teratogenic effects in the offspring (Huuskonen et al., 2008; Jedrychowski et al., 2013).

Bronchopulmonary dysplasia (BPD), which is characterized pathologically by diffuse alveolar enlargement, thinning of the septae and narrowing of bronchiolar diameters, also known as chronic lung disease of prematurity is the most common morbidity affecting premature babies with an incidence as high as 52% in extremely low birth weight (birth weights < 1000g) neonates (Natarajan et al., 2012). It also has long-term consequences such as chronic pulmonary morbidity, increased re-hospitalization rates, development of pulmonary hypertension and delayed neurodevelopment (Ambalavanan et al., 2011; Natarajan et al., 2012; Slaughter et al., 2011). Oxygen toxicity is thought to play a role in both acute lung injury and BPD. Prolonged exposure of newborn mice to hyperoxia, leads to lung pathology similar to human BPD (Warner et al., 1998). In critically ill patients,

hyperoxia may exacerbate or even cause acute lung injury. Exposure to hyperoxia postnatally is thought to contribute to the development of BPD in neonates (Vento et al., 2009). Hyperoxia leads to the production of reactive oxygen species (ROS) and these molecules lead to lung injury via oxidation of cellular macromolecules including DNA, protein and lipid (Freeman and Crapo, 1981). The molecular mechanisms by which hyperoxia causes lung injury are not understood, but CYP enzymes have been implicated (Hazinski et al., 1995). On the other hand, studies from our laboratory have demonstrated the protective effect of CYP1A enzymes against hyperoxic lung injury (Couroucli et al., 2011; Couroucli et al., 2002; Jiang et al., 2004; Moorthy et al., 2000; Sinha et al., 2005); Moorthy, 2008). However, there have been no studies on the effect of maternal exposure of environmental PAHs on hyperoxic lung injury in the offspring. Therefore, in this investigation, we tested the hypothesis that prenatal exposure of rats to the PAH BP will result in increased susceptibility of newborns to oxygen-mediated lung injury and alveolar simplification, and that CYP1A and 1B1 enzymes and oxidative stress mechanistically contribute to this phenomenon.

2. Materials and Methods

Animals

Thirteen days pregnant Fisher 344 rats were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and were divided into two groups. Purified tap water and rat chow (Purina Rodent Lab Chow No. 5001 from Purina Mills, Inc. (Richmond, Indiana) were made available to animals ad libitum. On day 18, 19 and 20 of pregnancy, one group (experimental) was administered i.p., 25mg/Kg/BW/day (w/v) BP dissolved in corn oil (CO) and another (control group) was administered only CO. Newborn rats delivered from mothers of both these groups were either maintained in room air or placed in oxygen chambers (85% O₂) immediately after birth for up to 7 days (first group) or 14 days (second group). All animal experiments were carried out in accordance to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. The experiments performed were reviewed and approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Hyperoxia Exposure

The newborn animals were either maintained in room air or exposed to 85% O₂ for 7 or 14 days whereas dams were rotated between hyperoxic and room air chambers once every 24 h to prevent oxygen toxicity to mothers.

Chemicals

Buffer components for electrophoresis and Western blotting were obtained from Bio-Rad Laboratories (Hercules, CA). The primary monoclonal antibody to CYP1A1, which cross-reacts with CYP1A2 was a generous gift from Dr. P. E. Thomas (Rutgers University, Piscataway, NJ), CYP1B1 (SC-32882) and β -actin(SC-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Goat anti-mouse IgG and Goat anti-rabbit IgG conjugated with horseradish peroxidase were from (Bio-Rad laboratories (Richmond, CA). BP(B1760-1G) was purchased from Sigma-Aldrich, Saint Louis MO. Antibody to anti-

mouse/anti-rat F4/80 (123102) for immunohistochemical analysis was purchased from BioLegend Inc, San Diego, CA, ECL reagent HyGlo (E5200) was purchased from Denville Scientific, Inc, Metuchen, NJ) and TRIzol reagent (15596018) was purchased from life Technologies, Carlsbad, CA

Tissue Perfusion and Harvesting

At the completion of exposure to hyperoxia for 7 or 14 days, the animals were sacrificed and their lungs and liver tissue were collected. The left lungs were fixed with zinc formalin by injecting formalin at a constant pressure (20cm of H₂O) through intratracheal catheter. Lungs were later embedded in paraffin for histological and immunohistochemical analyses. The right lungs were used for RNA isolation for qPCR, tissue lysate preparation for western blot analysis and F₂-isoprostane estimation. Tissue lysate of livers were also prepared for CYP1A1/1A2 and CYP1B1 analysis and RNA from liver was isolated for qPCR.

Preparation of Tissues for Histology

Paraffin embedded tissues were sectioned at 4 µm on a rotary microtome and were stained with hematoxylin and eosin (H&E) and anti- mouse/anti-rat F4/80 antibody. Stained sections were assessed for tissue morphology, injury and inflammation.

Western Blotting

Lung or liver tissue lysate were prepared from the tissues of individual animals in lysis buffer (0.25M Sucrose, 80mM Tris-HCl, 5mM MgCl₂, 1mM EDTA, 1mM DTT, 1mM PMSF and protease inhibitor cocktail tablet [(1 tablet in 10ml), pH-7.4]). Protein concentrations of the tissue lysate were determined by the Bradford method (Bio-Rad laboratories (Richmond, CA). Equal amount of total proteins were then resolved on SDS–10% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane was blocked in 5% nonfat skimmed milk and incubated with primary antibodies specific to CYP1A1, 1A2 or CYP1B1 overnight at 4°C followed by incubation with secondary antibodies for 1hr at room temperature. Proteins were visualized using ECL reagent as directed by the manufacturer. The densitometry of bands on western blot analysis was done using image J software and protein to β-actin densitometry ratio was analyzed using 2 way ANOVA followed by Bonferroni post-hoc tests to compare each group to all other groups using GraphsPad Prism5 software program. *P* values less than 0.05 were considered to be statistically significant.

Real time reverse transcriptase-polymerase chain reaction (RT-PCR) assays

Total RNA from the livers of rats was isolated using Trizol reagent. Sixty ng RNA from livers and 60 ng RNA from lungs of air-breathing or hyperoxic animals was subjected to one step real time quantitative TaqMan RTPCR using primers purchased from Applied Biosystems (CYP1A1: Rn00487218-m1; CYP1A2: Rn00561082-m1; CYP1B1: Rn00564055-m1 and 18s: Hs99999901) and detected using ABI PRISM 7700 Sequence Detection System. Each sample was analyzed in duplicate. The relative Ct values for CYP1A1, 1A2 and CYP1B1 were normalized to their 18S content and the relative expression levels of target genes were calculated according to the equation, 2^{-CT} , where

CT= Ct target gene– Ct control gene, as reported by us previously (Couroucli et al., 2006; 2011).

Analysis of F₂-isoprostanes

Levels of the F₂-isoprostane, 8-iso-prostaglandin F₂-alpha (8-iso-PGF₂α), which is a reliable biomarker for oxidative stress *in vivo* were determined in lung tissues by LC-MS essentially according to the protocol we reported recently (Couroucli et al., 2011). Rat lung tissues were suspended in methanol at a tissue wt/vol of 50 mg per mL of methanol (note: each mg of tissue was assumed to have a liquid volume of 1 μL or 58.1 mg of tissue = 58.1 μL of liquid volume). Lung tissue was placed into glass tube and mechanically homogenized. Five hundred μL of tissue homogenate was then transferred to microcentrifuge tube and centrifuged at maximum speed for 5 minutes at 5°C. Clarified supernatant was transferred to glass tube and liquid evaporated to dryness under a stream of nitrogen gas at room temperature. Dried residue was reconstituted with 50 μL of 50:50 methanol:0.2% acetic acid in water and transferred to LC sample tube for analysis by LC-MS. Analyses were performed using a Quattro Ultima mass spectrometer (Waters, Miford, MA) equipped with an Agilent 1100 binary pump HPLC system (Agilent, Wilmington, DE) 8-Iso-PGF₂α was chromatographically separated using a Luna 3 μm phenyl-hexyl 4.6×100 mm analytical column (Phenomenex, Torrance, CA). The mobile phase consisted of 10 mM ammonium acetate, pH 8.5 and methanol. A linear methanol gradient from 50% to 60% for 10 min and then from 60% to 80% in 4 min and further increased methanol concentration to 100% in 6 min and kept at that level for additional 2 min was used to achieve chromatographic baseline resolution for the isoprostanes of interest. The mass spectrometer was operated in negative electrospray ionization mode with constant voltage of 100 V. Source temperatures were 125 °C with desolvation gas temperature 350 °C. F₂-Isoprostanes were detected and quantified using multiple reaction mode (MRM) monitoring the transitions m/z 357.3→313.4 for 8-iso-PGF₂α-d₄, m/z 353.25→309.5 for 8-iso-PGF₂α

DNA adducts

DNA adducts derived from BP as well as oxidative DNA adducts were analyzed in genomic DNA of lungs of normoxic and hyperoxic newborn rats were analyzed by ³²P-postalbeling, as previously described (Reddy and Randerath, 1986). Briefly, DNA (10 μg) was enzymatically degraded to normal (Np) and modified (Xp) deoxyribonucleoside 3' monophosphates and their base-modified derivatives with a mixture of micrococcal endonuclease and spleen phosphodiesterase. After 3'-dephosphorylation of the normal nucleotides with nuclease P1, the enriched nuclease P1-resistant modified 3'-nucleotides were converted to 5'-³²P-labeled deoxyribonucleoside 3',5'-bisphosphate derivatives by incubation with carrier-free (γ-³²P)ATP and T4 polynucleotide kinase. The radioactively labeled adducts were separated by multidirectional anion-exchange thin-layer chromatography (TLC) on polyethyleneimine-cellulose sheets and chromatographed overnight (~15 hr) in the first direction (D1) with 2.3 M sodium phosphate, pH 5.75, to purify bulky adducts. The bulky labeled DNA adducts retained in the D1 chromatogram were contact-transferred to fresh thin-layer sheets and adducts were separated two-dimensionally with 3.82 M lithium formate, 6.75 M urea, pH 3.35, and 0.72 M sodium phosphate, 0.40 M Tris, 7.65 M urea, pH 8.20, for the first and second dimensions,

respectively (Zhou et al., 2011). Radioactive spots were located by imaging using an Instant Imager from Packard Instrument Co. (800 Research Parkway, Meriden, CT 06450) and by screen-enhanced autoradiography on Kodak XAR-5 X-ray film at -80°C for 22 h. The adducts were expressed as 1 in 10⁹ relative adduct labeling (RAL) values, as reported previously (Reddy and Randerath, 1986; Zhou et al., 2011).

Statistical Analyses

Statistical analyses for all experiments (with the exception of DNA adducts in Figure 8) were conducted using two-way ANOVA followed by Bonferroni post-hoc tests, which were performed using GraphPad Prism5 program software to compare each group to all other groups at both time points. Student t-test was used to compare DNA adducts in figure 8 as there were only two groups in each time point.

Results

Effects of BP or/and hyperoxia on lung inflammation and injury

Routine histological examination of the air breathing animals born to mothers who were administered only CO during pregnancy showed normal lung structure, and there was no evidence of tissue injury on PND 7 or 14 (Figs.1A a&b). After 7 days of hyperoxia the lungs showed pulmonary edema, alveolar hemorrhage and perivascular inflammation (Fig. 1A, c) which increased further after 14 days of hyperoxia, and we also observed underdeveloped septae (Fig.1A, d). On the contrary, air-breathing animals born to mothers who were administered BP during pregnancy showed some classical features of lung injury, exhibiting a moderate alveolar congestion, clusters of alveolar macrophages, stretching of alveolar walls and rupture of alveoli on PND 7 (Fig.1B, a). On PND 14, these animals also exhibited alveolar simplification and thickening of the alveoli (Fig.1B, b). Hyperoxic animals born to these mothers showed more severe injury as observed on PND 7 (Fig. 1B, c) as well on PND 14 (Fig. 1B, d), as compared to animals born to mothers who were administered only CO during pregnancy, but were exposed to hyperoxia (Figs. 1A c&d).

The extent of lung inflammation was assessed by immunohistochemistry of fixed lung sections to detect the expression of F4/80, which is a marker for macrophages (inflammatory cells) (Burns et al., 2012; Zaynagetdinov et al., 2013). Animals born to mothers administered only CO during pregnancy and were breathing room air, showed only very few macrophages on PND 7 or 14 (Figs.2A, a&b). However, newborn rats born to similar mothers but exposed to hyperoxia showed a significant number of macrophages on PND 7 and 14 (Figs.2A, c&d). Also, animals born to mothers who were prenatally administered BP, and breathing room air showed some macrophages (Figs. 2B, a&b), whereas newborn rats exposed to hyperoxia showed much greater number of macrophages in the lungs on PND 7 as well as PND 14 (Figs. 2B, c&d).

Effect of prenatal BP administration on *Cyp1a/1b1* gene expression in the liver of newborn rats breathing room air or exposed to hyperoxia

On PND 14, markedly higher level of *Cyp1a1* gene expression (~35-fold) was found in the livers of animals born to mothers exposed to BP and breathing air, as compared to animals

born to mothers exposed to CO only during pregnancy and breathing air, or to animals born to mothers administered BP during pregnancy and exposed to hyperoxia (Fig.3A). The expression levels of *Cyp1a2* gene were also significantly higher in air-breathing rats that were prenatally exposed to BP, as compared to those that were prenatally treated with CO (Fig. 3B). The induction of *Cyp1a1* as well as *Cyp1a2* genes declined to control levels in rats exposed to hyperoxia for 14 days. No significant differences in the gene expression of CYP1A1, 1a2, or 1b1 among any of the groups was observed at PND 7 (data not shown). Significant differences in the mRNA expression levels of any of these genes were also not observed in the lungs of the animals treated with various regimens (data not shown).

Effect of prenatal BP administration on Cyp1 protein expression in the livers of newborn rats breathing room air or exposed to hyperoxia

There was no significant difference found in the PND 7 or PND 14 in the CYP1A1/A2 protein levels in any of the groups (Figs.4A a&b). However, CYP1B1 protein levels were significantly upregulated on PND7 in hyperoxic animals that were maternally exposed to BP, as compared to animals that were hyperoxic or air-breathing animals that were maternally exposed to CO (Figs.4A, a and 4B, b).

Effect of prenatal BP administration on CYP1A/1B1 protein expression in lungs of newborn rats breathing room air or exposed to hyperoxia

The CYP1A1 protein levels were found to be significantly high in the lungs on PND 7 in the hyperoxic animal group born to mothers administered BP maternally, as compared to the group born to mothers exposed to CO only (Figs. 5A, a and 4B, a). On PND14, the CYP1A1 levels were significantly decreased in the animals born to mothers exposed to BP during pregnancy. The animal group breathing air or hyperoxia did not show any significant difference in its expression levels (Figs. 5A a&b).

The CYP1B1 protein levels were found to be significantly low on PND 7 in hyperoxic animal group born to mothers exposed to CO during pregnancy (Figs. 5A,a and 5B, b) compared to air breathing group. On the contrary, CYP1B1 protein levels were significantly up-regulated in hyperoxic animals born to mothers exposed to BP during pregnancy (Figs. 5A,a and 5B, b). On PND 14, CYP1B1 levels were lower in animals born to mothers administered BP and exposed to hyperoxia than the group born to mothers administered CO and exposed to hyperoxia (Figs. 5A, b and 5B, b).

Effect of BP and/or hyperoxia on F₂-isoprostane, 8-iso-PGF₂α, a marker of lipid peroxidation in lungs

To assess the oxidative stress-induced lipid peroxidation, 8-iso-PGF₂α was measured in the lung tissues. There were no significant differences found in the level of 8-iso-PGF₂α in the various groups irrespective of those breathing air or are exposed to hyperoxia and born to mothers administered either BP or corn-oil at PND 7. However, on PND 14 animals exposed to hyperoxia and born to mothers administered BP during pregnancy had significantly higher levels of 8-iso-PGF₂α, as compared to animals exposed hyperoxia but born to mothers not administered BP during pregnancy (Fig.6).

Effect of prenatal BP administration on DNA adducts in lungs of newborn rats breathing room air or exposed to hyperoxia

In newborn rats that were treated prenatally with BP, BP-derived DNA adducts were observed in lungs of normoxic as well as hyperoxic rats, and these adducts were not formed in rats that were prenatally exposed to CO (Figure 7). In newborns that were maternally exposed to BNF (Couroucli et al., 2011), BNF-DNA adducts were not detected (Figure 7). Interestingly, hyperoxia by itself induced oxidative DNA adducts, and also significantly augmented formation of BP-DNA adducts (Figure 8).

3. Discussion

In this investigation, we tested the hypothesis that prenatal exposure of rats to the PAH BP will result in increased susceptibility of newborns to oxygen-mediated lung injury and alveolar simplification, and that oxidative stress in part contributes to this effect. The increases in lung injury and inflammation in hyperoxic mice born to CO-treated mothers, compared to air-breathing controls (Figures 1 and 2) were in agreement with the findings of other investigators (Bhandari, 2010; Crapo, 1986; Warner et al., 1998). In fact, studies of hyperoxic chronic injury in newborn rats have shown morphologic changes similar to those seen in human BPD (Bhandari, 2010) and extremely premature baboons (Coalson et al., 1999). The marked potentiation of hyperoxia-mediated lung damage in newborn rats that received BP (25 mg/kg) transplacentally (on gestational days 17-19) supported the hypothesis that this compound exacerbates hyperoxic lung injury. In fact, our observation that prenatal BP caused augmentation of lung injury in newborns maintained in room air (Figure 1) is intriguing, and suggests that maternal BP makes the offspring highly susceptible to oxidative stress. The rationale for giving BP during gestational days 17-19 was that gestational days 17-19 represent the canalicular and saccular stages of fetal lung development, and this corresponds to a gestational weeks of 16-27 weeks in humans (Bhattacharya and Mariani, 2013). Because premature infants born between 22-27 weeks are at high risk for developing bronchopulmonary dysplasia (BPD), we administered BP on gestational days 17-19 in order to determine the impact of maternal BP on hyperoxic lung injury and abnormal lung maturation in the newborn rats.

The reason for selecting 25 mg/kg BP for 3 days was on the basis of previous studies that treatment of pregnant mice with a single i.p., dose of the PAH 3-methylcholanthrene (45 mg/kg) on gestational day 17 resulted in marked fetal induction of CYP1A1 and 1B1, which persisted for up to 48 h (Miller and Marty, 2010; Xu and Miller, 2004; Xu et al., 2005). In our previous study (Couroucli et al., 2011), we treated pregnant mice with BNF (40 mg/kg) on gestational days 17-19, and we observed significant fetal induction of hepatic and pulmonary CYP1A1. In our current study, we treated pregnant rats with BP on days 17-19 with 25 mg/kg (total accumulated dose of 75 mg/kg) as BP is less potent than 3-methylcholanthrene as a CYP1A1 inducer, and our goal was to test if fetal induction of pulmonary CYP1A1 enzyme will lead to modulation of hyperoxic lung injury in newborn rats.

Our observations that macrophage infiltration in the lungs was substantially augmented in newborn rats, which had been prenatally treated with the vehicle CO, and exposed to

hyperoxia for 7-14 days (Figs. 2A, B) was probably due to increased inflammation in these rats. The marked potentiation of macrophages in hyperoxic rats that had been prenatally treated with BP supported the hypothesis that BP augments lung inflammation induced by hyperoxia. In fact, maternal BP also enhanced macrophage recruitment in newborn rats maintained in room air, supporting the hypothesis that BP administered prenatally makes the offspring more susceptible to lung inflammation after birth, and this was most likely due to increased oxidative stress.

The robust induction of hepatic CYP1A1 and 1A2 gene expression at 14 PND (Fig. 3A &B) in air-breathing newborn rats treated prenatally with BP indicated that the BP-mediated effects were due to transplacental transfer of BP from the mother to fetus, resulting in the induction of CYP1A1 and 1A2. Transplacental induction of CYP1A1 by the PAH 3-methylcholanthrene (MC) has been shown previously (Anderson et al., 1989a; Anderson et al., 1991; Anderson et al., 1989b; Miller, 1994; Xu et al., 2005). Recently, we reported marked protection against hyperoxia-mediated lung damage in newborn mice that received BNF, which also transplacentally induced CYP1A1 in liver and lung (Couroucli et al., 2011). It is not clear as to why the induction of gene expression of CYP1A1 and 1a2 was not accompanied by concomitant induction of the corresponding protein. It is possible that transplacental transfer of reactive metabolites from BP in the newborn may have caused decline of Cyp1a proteins through covalent binding. On the other hand, maternal BP induced CYP1B1 protein at PND 7 (Figs. 4A,a and 4B, b). The marked induction of CYP1B1 in rats prenatally exposed to BP may have in part contributed to the augmented inflammation. This interpretation is based on the studies reported by Smerdova et al. (2013) who showed that CYP1B1 is elevated in cells under inflammatory conditions.

The significant induction of CYP1A1 protein in lungs of hyperoxic rats prenatally exposed to BP on PND7, compared to hyperoxic or normoxic rats that were prenatally treated with CO (Figs. 5A,a and 5B,a) was probably due to transplacental induction of pulmonary CYP1A1 by BP as discussed above. The decline of induction of CYP1A1 in normoxic as well as hyperoxic rats that were prenatally exposed to BP at PND 14 (Figs. 5A,b and 5B,a) was probably due to the metabolism and clearance of BP by PND 14.

The induction of pulmonary CYP1B1 in hyperoxic rats maternally exposed to BP on PND 7 (Figs. 5A,a and 5B,b) could be explained by increased expression of this protein under inflammatory conditions (Smerdova et al., 2013). The decline of induction of CYP1B1 in hyperoxic rats prenatally exposed to BP compared to those prenatally exposed to Co (Figs. 5A,b and 5B,b) may have been due to increased oxidative stress that may have resulted in attenuation of CYP1B1 expression. This interpretation is based on the increased levels of 8-iso-PGF2 α in lungs on PND 14 of hyperoxic rats that were prenatally exposed to BP (Fig. 6B).

Recent studies have provided evidence for the involvement of lipid peroxidation products derived from arachidonic acid, i.e. F₂-isoprostanes and isofurans in mediating lung injury mediated by hyperoxia (Fessel et al., 2002; Janssen, 2000; Morrow and Roberts, 2002; Zahler and Becker, 1999). These molecules have also been implicated in the development of lung injury associated with BPD (Goil et al., 1998; Saugstad, 1997). Our observations (Fig.

6) showing increased levels of the 8-iso-PGF₂α in lungs of on PND 14 of hyperoxic rats that were prenatally exposed to BP (Fig. 6B) supported the hypothesis that 8-iso-PGF₂α plays a mechanistic role in the development of lung injury.

We reported earlier that mice treated prenatally with the CYP1A inducer β-naphthoflavone (BNF) were less susceptible to hyperoxic lung injury than those prenatally exposed to CO (Couroucli et al., 2011). Although BP is a CYP1A inducer, our observation that prenatal exposure of BP potentiated hyperoxic lung injury and alveolar simplification in newborn rats could have been due to the genotoxic effects of BP in the offspring Urso et al., 2010; Herbstman et al., 2012). Thus while BP and BNF are able to transplacentally induce CYP1A/1B enzymes, the major difference between these two compounds is that BP is genotoxic (Figure 7), while BNF is non-genotoxic (Figure 7), and this may in part explain the opposing effects of these compounds on the susceptibility of the offspring to hyperoxic lung injury and alveolar simplification upon maternal exposure. Furthermore, newborn rats that were exposed prenatally to BP displayed increased formation of BP-derived as well as oxidative DNA adducts (Figures 7 and 8) that were not observed in animals prenatally exposed to BNF (Figure 7), lending further credence to the hypothesis that combined genotoxic effects of BP and hyperoxia contributed to the increased susceptibility of rats to lung injury. (Lu et al., 1986) showed that treatment of pregnant mice with BP (50 mg/kg), on gestational day 18 leads to formation of maternal and fetal DNA adducts, supporting the hypothesis that transplacental exposure BP elicits genotoxicity in the newborn. It is also possible some of the effects of BP on hyperoxic lung injury may have been due to transplacental epigenetic effects (Herbstman et al., 2012), but further studies are needed to provide support for this hypothesis.

In conclusion, our results show increased susceptibility of newborns to oxygen-mediated lung injury and alveolar simplification following maternal exposure to BP, and our results suggest that modulation of CYP1A/1B1 enzymes, increases in oxidative stress, and BP-DNA adducts contributed to this phenomenon. These studies have important implications for chronic lung disease in infants and provide opportunities for conducting mechanistic studies in understanding the link between maternal exposure to environmental pollutants and oxygen-mediated lung diseases such as BPD in infants.

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Abbreviations

BPD	Bronchopulmonary dysplasia
PAH	Polycyclic aromatic hydrocarbon
BP	Benzo[a]pyrene (BP)
CYP	Cytochrome P450
ANOVA	Analyses of variance
RT-PCR	Reverse transcriptase Polymerase Chain Reaction
CO	Corn oil
PND	Postnatal day
ROS	Reactive oxygen species
LW/BW	Lung weight/Body weight
8-iso-PGF2α	8-iso-prostaglandin-F2-alpha
RAL	Relative adduct labeling

Highlights

- !! Prenatal benzo[a]pyrene (BP) potentiated hyperoxic lung injury in newborn rats
- !! Prenatal BP exacerbated alveolar simplification and inflammation in newborn rats
- !! Maternal BP and neonatal hyperoxia significantly modulated CYP1 enzyme expression
- !! Oxidative stress contributed to the BP-mediated effects on newborn lung

Figure 1. Hematoxylin/eosin-stained images from the lungs of rats

Neonatal rats born to mothers administered BP show greater alveolar simplification compared to control rats with no prenatal exposure. Representative hematoxylin/eosin-stained images from the lungs of rats **A.** without prenatal BP (Maternal CO: CO) or **B.** with prenatal BP (Maternal BP) exposure. The top left panels (A,a&b) show the maternal CO group exposed to air and top right panels shows the maternal CO group exposed to 85% oxygen for 7 or 14 PNDs respectively (A,c&d). The bottom left panels show the maternal

BP exposure group exposed to air (B,**a&b**) and bottom right panels show the maternal BP exposure group exposed to 85% oxygen for 7 and 14 PNDs respectively (B,**c&d**).

Figure 2. Representative immuno-stained images for macrophages in the lungs

Macrophage recruitment into the lungs was determined by immunohistochemistry with anti-F4/80 antibody in neonatal rats exposed to prenatal BP compared to control rats with no prenatal exposure. The top left panels (a&b) show the maternal CO group exposed to air and top right panels shows the maternal CO group exposed to 85% oxygen for 7 or 14 PND, respectively (c&d). The bottom left panels show the the maternal BP exposure group exposed to air (a&b) and bottom right panels shows the maternal BP exposure group exposed to 85% oxygen for 7 and 14 PND, respectively (c&d) Scale bar: 200 microns. Arrows point to macrophages in the lung.

Figure 3. Real-time (RT-PCR) analysis showing the effects of prenatal BP exposure and hyperoxia exposure on hepatic CYP1A1 (A) and CYP1A2 (B) for 14 days
mRNA expression in neonatal rats exposed to prenatal BP compared to control rats with no prenatal exposure. Values are fold change in mRNA expression over expression levels in room air controls from at least three individual animals. *P* value of <0.05 was considered significantly different.

Figure 4. The expression of CYP1A1/1A2 and CYP1B1 protein levels in the liver of rats exposed to normal air or hyperoxia (85% oxygen) and born to mothers administered intraperitoneal BP during pregnancy

Pups born to pregnant rats which were administered with BP at a dose of 25mg/Kg/BW/day (w/v) dissolved in CO (as a vehicle) or CO alone on 18, 19 and 20 days of pregnancy were divided into four groups each soon after birth and were exposed to either room air or hyperoxia (85% oxygen) for 7(a) or 14(b) days. **A.** Expression of CYP1A1/a2 and CYP1B1 proteins was determined by western blot analysis using β -actin as loading control. **B.** Using Image J imaging program, density of the bands in Western blot analysis was measured and expressed as mean $\pm$ SEM of protein to β -actin ratio. *P*-value of less than 0.05 was considered statistically significant.

Figure 5. Effect of BP administration to pregnant rats on the protein expression levels of CYP1A1 and CYP1B1 in the lungs of newborns exposed to normal air or hyperoxia (85% oxygen)

BP (dissolved in CO) or CO alone was administered i.p to pregnant rats at 25 mg/Kg/BW/day (w/v) dose on day 18, 19 and 20 of pregnancy. Pups born to these rats were divided soon after birth into four groups each and were exposed to either room air or hyperoxia (85% oxygen) for 7(a) or 14(b) days. **A.** Protein expression of CYP1A1 and CYP1B1 was determined by western blot analysis using β -actin as a loading control. **B.** Densitometric measurement of the bands in Western blot analysis was performed using Image J software and the results were expressed as mean $\pm$ SEM of protein to β -actin ratio. *P*-value less than 0.05 was considered to be statistically significant.

Figure 6. Lipid peroxidation

Quantitative analysis of the effects of hyperoxia exposure on lipid peroxidation in neonatal rats exposed to prenatal BP compared to control rats with no prenatal exposure. **(A)**: shows pulmonary F₂-isoprostanes in neonatal rats exposed to 85% oxygen for 7 days postnatally and **(B)** exposed to 85% oxygen for 14 days postnatally. Values are means $\pm$ S.E.M. from at least three individual animals. Significant differences are indicated by * $p < 0.05$.

Figure 7. DNA adducts

Representative profiles of ^{32}P -postlabelled DNA adducts, in lung DNA of normoxic (air) or hyperoxic (O_2) newborn rats that were prenatally administered CO or BP, were analyzed at the indicated time points by the ^{32}P -postlabeling assay, as described in Materials and Methods. Screen-enhanced autoradiography employed Kodak XAR-5 film at 80°C for 22 hours. Circled adducts 1-4 in rats maintained in room air (panel A) were background spots, and adducts 5 and 6 (panel B) probably representative of oxidative DNA adducts, as they were specifically induced in hyperoxic rats. Spots a, b, c, and d (Panels C, D, E, and F) were BP-derived DNA adducts, as they were not present in rats that were prenatally exposed to CO. Spots 7, 8, and 9 (panel D) were induced by a combination of hyperoxia and BP, as they were not formed in normoxic rats or in hyperoxic rats that were maternally exposed to CO. Panel E and F represent ^{32}P -postlabeling profile of adducts from lungs of normoxic (panel E) or hyperoxic (panel F) that were prenatally exposed to BNF, and newborns were sacrificed on PND 5 (Courouclis et al, 2011).

Figure 8.

Quantitative analyses of pulmonary DNA adducts in mice treated prenatally with BP or CO. DNA adducts were analyzed in lungs of hyperoxic newborn rats that were exposed prenatally to CO or BP, as described in legend to figure 7, and adducts spots were located by imaging using an Instant Imager from Packard Instrument. The adducts were expressed as 1 in 10⁹ relative adduct labeling (RAL) values, as described in Materials and Methods. Values represent mean $\pm$ SEM of total adduct values (BP-DNA + oxidative DNA adducts) in DNA of hyperoxic newborn rats at PND 7 or 14 from at least 3 individual animals. *, Statistically significant at $P < 0.05$ between prenatal BP and CO groups by Student's t-test.