Published in final edited form as:

Toxicology. 2015 May 4; 331: 14–23. doi:10.1016/j.tox.2015.01.019.

Sex-specific differences in hyperoxic lung injury in mice: role of Cytochrome P450 (CYP)1A

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Abstract

Sex-specific differences in pulmonary morbidity in adults and preterm infants are well documented. Hyperoxia contributes to lung injury in experimental animals and humans. Cytochrome P450 (CYP)1A enzymes have been shown to play a mechanistic role in hyperoxic lung injury (HLI) in animal models. Whether CYP1A enzymes contribute to gender-specific differences in relation to HLI is unknown. In this investigation, we tested the hypothesis that mice will display gender-specific differences in HLI, and that this phenomenon will be altered in mice lacking the genes for *Cyp1a1* or *1a2*. Eight week-old male and female wild type (WT) (C57BL/6J) mice, *Cyp1a1*−/−, and *Cyp1a2*−/− mice were exposed to 72 hours of hyperoxia (FiO_2>0.95). Lung injury and inflammation were assessed and pulmonary and hepatic CYP1A1 and CYP1A2 levels were quantified at the enzyme activity, protein and mRNA level. Upon exposure to hyperoxia, liver and lung microsomal proteins showed higher pulmonary CYP1A1 (apoprotein level and activity) in WT females compared to WT males and a greater induction in hepatic CYP1A2 mRNA levels and activity in WT females after hyperoxia exposure. The gender based female advantage was lost or reversed in *Cyp1a1*−/− and *Cyp1a2*−/− mice. These findings suggest an important role for CYP1A enzymes in the gender-specific modulation of hyperoxic lung injury.

Keywords

Hyperoxia; Sex-specific; CYP1A; Lung injury

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1. Introduction

Sex-specific differences have been documented to play a role in the pathogenesis and outcome in various diseases in adults and children. Acute respiratory distress syndrome (ARDS) is a devastating clinical disorder in critically ill patients that causes acute lung injury and has a high mortality. Mortality in ARDS is higher in males compared to females (Moss and Mannino, 2002). Oxygen toxicity plays a role in both acute lung injury and ARDS. Hyperoxia may exacerbate or even cause acute lung injury (ALI) in mechanically ventilated patients. It leads to the production of reactive oxygen species that can in turn lead to lung injury via oxidation of cellular macromolecules including DNA, protein and lipid (Freeman and Crapo, 1981; Moss and Mannino, 2002).

The Cytochrome P450 (CYP) enzymes belong to a super family of hemeproteins, involved in the metabolism of exogenous and endogenous chemicals (Guengerich, 2004). Several groups including ours have demonstrated the protective effect of CYP1A in hyperoxic lung injury (HLI) (Lingappan et al., 2014). The CYP1A subfamily comprises 2 isoforms, i.e. CYP1A1 and 1A2. CYP1A1 is essentially an extra-hepatic enzyme (rodent and human lung, intestine, placenta and kidney) while CYP1A2 is expressed mainly in the rodent and human liver. Animals exposed to hyperoxia display upregulation of pulmonary and hepatic CYP1A1 and 1A2 expression for up to 48 hours, and by 60 hours these animals show overt respiratory distress (Couroucli et al., 2002; Moorthy et al., 1997). CYP1A inducers like beta-naphthoflavone (BNF) (Mansour et al., 1988) or 3-methylchloanthrene (3-MC) (Sinha et al., 2005) attenuate and CYP1A inhibitors like 1-aminobenzotriazole (Moorthy et al., 2000) potentiate HLI in rodents. The induction of CYP1A is thought to occur through the Aryl hydrocarbon receptor (AhR) pathway. Aryl hydrocarbon dysfunctional (AhRd) mice were more susceptible to HLI compared to wild type controls (Jiang et al., 2004). Prenatal administration of CYP1A inducer BNF attenuates HLI in newborn mice (Couroucli et al., 2011). These observations support the theory that CYP1A1 and CYP1A2 may play protective roles against oxygen-mediated lung injury.

The impact of sex and sex hormones on lung physiology and disease has been extensively studied in animal models. Gender also contributes to differential lung development prenatally and has a major role in the development of disease conditions from the neonatal (respiratory distress syndrome) to the adult period (asthma, lung cancer, interstitial lung disease) (Carey et al., 2007; Casimir et al., 2013). This is probably due to modulation by sex hormones, which may contribute to the disease pathogenesis or serve as protective factors, depending on the disease. With respect to acute lung injury due to hyperoxia, Neriishi et al (Neriishi and Frank, 1984) showed that castration prolonged tolerance of young male rats to pulmonary O_2 toxicity. In other acute lung injury models, testosterone was found to increase (Card et al., 2006) and estrogen to ameliorate inflammation and injury (Speyer et al., 2005). We have shown that WT male mice are more susceptible than females to hyperoxic lung injury and that, differences in inflammatory and oxidative stress markers contribute to these gender-specific dimorphic effects (Lingappan et al., 2013).

Several endogenous factors, including hormones, can modify CYP1A induction, directly influencing CYP1A gene expression or indirectly via cross talks with other transcription

factors (Monostory et al., 2009). Molecules with steroidal structure can be AhR ligands and may induce CYP1A expression in an AhR-dependent manner. Previous studies have shown that estradiol and progesterone induce CYP1A1 activity and testosterone on the other hand decreases CYP1A expression (Kojima et al., 2008; Lee et al., 1998; Monostory et al., 2009). Sex related differences in the constitutive expression of CYP1A have also been observed in animal models (Kojima et al., 2008; 2010; Rasmussen et al., 2011).

The gender-based dimorphic response in lung injury due to hyperoxia and the role of the CYP1A subfamily using knock out mouse models has not been studied. In this investigation, we tested the hypothesis that the gender-based differences in hyperoxic lung injury as observed in WT mice, will be lost in mice lacking the genes for *Cyp1a1*or *Cyp1a2*.

2. Materials and Methods

2.1. 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 back crossed with C57BL/6J mice for 12 generations to generate theoretically a >99.2% C57BL/6J background. Breeding pairs of *Cyp1a1*−/− mice of C57BL/6J background were obtained from Dr. D.W. Nebert (University of Cincinnati Medical Center, Cincinnati, OH). Eight-week old male and female C57BL/6J wild type (WT), *Cyp1a1*−/− and *Cyp1a2*−/− mice were maintained at Texas Children's Hospital animal facility and used for the study. Both *Cyp1a1*−/− and *Cyp1a2*−/− mice are phenotypically similar to the WT mice and have normal growth (Dalton et al., 2000; Peterson et al., 2004). They 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.

2.2. Chemicals

Tris, sucrose, NADPH, bovine serum albumin, ethoxyresorufin, methoxyresorufin were purchased from Sigma Chemical Co. (St. Louis, MO). 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 obtained from Dr. P. E. Thomas (Rutgers University, Piscataway, NJ). Goat anti-mouse IgG conjugated with horseradish peroxidase was from Bio-Rad laboratories (Richmond, CA). All Real Time RT-PCR reagents were from Applied Biosystems (Foster City, CA).

2.3. Protocols

Briefly, we used a total of 10 animals per sex per genotype (WT, *Cyp1a1*−/− and *Cyp1a2*−/−) in the study. The mice were then maintained in either room air (21% oxygen) in standard laboratory mouse cage or exposed to hyperoxic (95–100% oxygen) environment using pure O_2 at 5 l/min for 24 , 48 and 72 h in a sealed Plexiglas chamber, as reported previously

(Gonder et al., 1985). After sealing, the oxygen concentration in the Plexiglas chamber was checked frequently by an analyzer (Getronics, Kenilworth, New Jersey). Purified tap water and food (Purina Rodent Lab Chow 5001 from Purina Mills, Inc., Richmond, IN) were available *ad libitum*. After hyperoxia exposure, the animals were immediately anesthetized with sodium pentobarbital (200 mg/kg i.p.) and euthanized by exsanguination while under deep pentobarbital anesthesia. Three animals from each group were used for lung histopathology and from the seven remaining animals in each group; lung and liver tissues were harvested for the isolation of mRNA, protein, and microsomes. Lung weight and body weight, histology, immunohistochemistry for neutrophil infiltration was reported at 72 h as the effect of hyperoxia on earlier time points was not significant as previously reported (Moorthy et al., 1997). mRNA expression was assessed at earlier time points as transcription usually precedes changes in protein levels.

2.4. Lung weight and body weight (BW) measurements

The mice were weighed immediately after being anesthetized. Lung weights were measured after sacrifice and harvesting to evaluate the severity of lung edema.

2.5. Preparation of tissues for histology, histopathology and immunohistochemistry for neutrophil infiltration

A separate group of animals were used for these analyses. Lungs were fixed for histological analysis. Liver tissue from these animals was harvested for further downstream protein and RNA extraction. Tracheotomy was performed on the anesthetized mice and the lung tissue was fixed by intratracheal instillation of 10% zinc formalin at constant pressure of 25 cm of H_2O (Couroucli et al., 2002). Samples were left in solution for 24 h in 10% zinc formalin, and then transferred to 70% ethanol for long-term storage. Routine histology was performed on lung tissues from individual animals following staining of the paraffin sections with hematoxylin and eosin. For quantification of lung injury we used the guidelines published in the American Thoracic Society workshop report for the measurement of acute lung injury in experimental animals (Matute-Bello et al., 2011). Briefly, 20 random high-power fields were independently scored in a blinded fashion for five histological findings: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces and alveolar septal thickening were graded using a three tiered schema resulting in a score between zero and one. For assessment of neutrophil infiltration, 5 μM deparaffinized lung sections were immunostained with rat anti-mouse neutrophil antibody (Serotec, Raleigh, NC; MCA771G, dilution 1:200) for neutrophils, followed by staining with biotinylated secondary antibodies (Vector Laboratories Burlingame, CA). To analyze the degree of pulmonary neutrophil infiltration, the positively stained cells were counted in 20 non-adjacent areas per mouse under 40x magnification. A pulmonary pathologist, who was blinded to the treatment of mice with various regimens, evaluated the histopathology and immunohistochemistry slides.

2.6. Preparation of microsomes and enzyme assays

Lung and liver samples at the time of dissection were frozen immediately with liquid nitrogen and maintained at a temperature of – 80°C until preparation of microsomes. Liver

microsomes were isolated by the calcium chloride precipitation method (Moorthy et al., 1997). Lung microsomes were isolated by differential centrifugation, as published previously {Matsubara:1974ty}. Protein concentration was measured by the Bradford dye binding method. Ethoxyresorufin *O*-deethylase (EROD) (CYP1A1) activities in lung and liver microsomes and methoxyresorufin *O*-demethylase (MROD) (CYP1A2) activities in liver microsomes were assayed as described previously (Moorthy et al., 1997).

2.7. Western blotting

Liver microsomes (10 μg of protein) prepared from individual animals were subjected to SDS polyacrylamide gel electrophoresis in 10% acrylamide gels. The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by western blotting. For the western blot analysis, a monoclonal antibody to CYP1A1, which cross-reacts with CYP1A2 was used as a primary antibody. The primary antibody was detected by incubation with the horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody. Glucose-regulated protein (GRP, 78 kDa), a microsomal protein was used as the housekeeping protein. Lung whole protein (20 μg of protein) prepared from individual animals in Milliplex MAP lysis buffer (EMD Millipore) was used for apoprotein estimation. This was done at 48 h, because at 72 h there was significant protein degradation in lung isolates. For loading controls, the membranes were stripped and incubated with antibodies against β-actin, followed by electrochemical detection of bands.

2.8. Real Time RT-PCR assays

Total mRNA was isolated using a modification of the procedure from Chomczynski *et al.* (Chomczynski and Sacchi, 1987)and treated with RQ1 RNase-free DNase (Promega) to eliminate genomic DNA contamination. RNA (50 ng), isolated as above, was subjected to one step real time quantitative TaqMan® RT-PCR using 7900HT Fast Real- Time PCR System (Applied Biosystems, Foster City, CA). Gene-specific primers (CYP1a1-Mm00487217_m1; TNF-α Mm00443258_m1; 18SrRNA-Hs99999901_s1) in the presence of TaqMan® reverse transcription reagents and RT reaction mix (Applied Biosystems, Foster City, CA) were used to reverse transcribe RNA, and TaqMan®Gene Expression probes and TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) were used for PCR amplification. For CYP1A2, the forward primer was: TCCTGGACTGACTCCCACAAC and the reverse primer was: GAACGCCATCTGTACCACTGAA. 18SrRNA was used as the reference gene. Following an RT hold for 30 minutes at 48°C, the samples were denatured at 95°C for 10 minutes. The thermal cycling step was for 40 cycles at 95°C for 15 s, and 40 cycles at 60°C for 1 minute. The ΔΔCt method was used to calculate the fold change in mRNA expression: ΔCt = Ct (target gene)- Ct (reference gene), ΔΔCt = ΔCt (treatment) - ΔCt (control), fold change = $2^{(-\Delta\Delta Ct)}$ (Jiang et al., 2004).

2.9. Data analysis

The comparison between male and female mice, the three genotypes, and the effect of hyperoxia exposure was done using two-way ANOVA, followed by Bonferroni post-hoc

tests, and $p \leq 0.05$ was considered significant. The statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA)

3. Results

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

Figure 1A shows the effect of hyperoxia on body weight of male and female animals after exposure to hyperoxia. WT females showed no significant weight loss similar to our previous findings (Lingappan et al., 2013). The weight loss was significant in all other groups and was more significant in males than females. Figure 1B represents the fold change in lung weight from respective room air controls after exposure to hyperoxia. Since differences existed in lung weights between male and female animals even at room air conditions, we expressed the ratio as fold change after exposure to hyperoxia (72 h) compared to the corresponding room air breathing control animals. Exposure to hyperoxia increased the lung weights in all the groups. *Cyp1a1* and *Cyp1a2*−/− animals showed gender-specific changes with females showing greater lung weight after 72 hours of hyperoxia exposure. The increases in lung weights were also greater in *Cyp1a1*−/− and *Cyp1a2* −/− females when compared to respective WT females and in *Cyp1a2*−/− males when compared to WT males.

3.2. Lung histopathology

Histopathological examination of the lung sections after exposure to hyperoxia for 72 h showed greater perivascular, bronchiolar edema and alveolar hemorrhage in male WT mice when compared to female mice (Fig. 2A and 2B). The room air control images from the animals are shown in Supplementary figure 1. There was no difference in lung histology between the genotypes at baseline. In *Cyp1a2*−/− mice, females showed more signs of lung injury when compared to males. Lung injury was similar among male and female mice in *Cyp1a1*−/− mice. This was quantified objectively (Figure 3) using the American Thoracic Society guidelines for the measurement of acute lung injury in experimental animals as described in Materials and Methods (Matute-Bello et al., 2011). After 72 h of hyperoxia exposure, WT males showed greater lung injury compared to females and the reverse was observed in *Cyp1a2*−/− animals. There were no differences between males and female mice in the *Cyp1a1* −/− group. Loss of CYP1A (1A1 or 1A2) led to increased lung injury in both males and female animals, with females showing a more significant effect.

3.3. Neutrophil Infiltration

In order to assess whether increased lung injury after hyperoxia is accompanied by exacerbation of inflammation, we determined the extent of neutrophil recruitment in lung of mice exposed to hyperoxia. Upon exposure to hyperoxia for 72 h, lungs showed neutrophil infiltration in response to the injury as shown in Figure 4. Representative images from room air controls and animals exposed to hyperoxia are shown. Figure 5 shows the quantitative assessment of neutrophil infiltration in the lungs. There was no difference at room air conditions. After hyperoxia exposure, WT males showed higher neutrophil infiltration in the lungs when compared to females (Fig 5) ($P<0.05$). There were no differences among *Cyp1a1*−/− animals between males and females. Among *Cyp1a2*−/− animals, female mice

showed significantly higher neutrophil infiltration compared to males after exposure to hyperoxia ($p<0.05$). Similar to the results with lung injury, there was significantly more neutrophil infiltration in *Cyp1a1*−/− and *Cyp1a2*−/− animals than WT mice.

3.4. TNF- α mRNA expression in the lungs after hyperoxia exposure

Figure 6 shows the expression of TNF-α mRNA in the lungs after 24, 48 and 72 h of hyperoxia expressed as fold change over room air levels. Figure 6A, 6B and 6C shows the trend of TNF-α mRNA between male and female mice in WT, *Cyp1a1*−/− and *Cyp1a2*−/− mice, respectively. Among the *Cyp1a2*−/− mice, females showed a significantly higher expression at 24 h ($P<0.05$) compared to males. WT males displayed higher expression at 72 h compared to females but this trend was not statistically significant. *Cyp1a2*−/− male mice showed increased expression at 48 ($P<0.01$) and 72 h ($P<0.001$) compared to room air levels. WT and *Cyp1a1*−/− males showed increased expression ($P<0.05$) at 72 hours compared to room air controls. *Cyp1a2*−/− female mice (Figure 6B) had increased expression at 24 h ($P<0.001$), 48 h ($P<0.01$) and 72 h ($P<0.001$) compared to room air controls. There were no differences in the baseline expression of TNF-α between any of the groups. *Cyp1a2*−/− mice of either sex showed increased expression compared to WT mice (data not shown).

3.5. Effect of hyperoxia on hepatic CYP1A activity and expression

Hepatic CYP1A2 enzyme activity was measured using the MROD assay. Female mice (WT and *Cyp1a1*−/−), showed higher CYP1A2 enzyme activity as measured by the MROD assay (Fig 7A) after exposure to hyperoxia ($p<0.01$) compared to male mice. The decline in CYP1A2 activity compared to baseline room air levels after 72 h hyperoxia exposure was significant in male mice (WT and *Cyp1a1*−/−) ($p <0.01$), and was better preserved in female mice. Next, we determined CYP1A protein expression in the liver microsomal protein fraction under room air and after hyperoxia exposure (72 h) in WT and *Cyp1a1*−/− animals. Western blot assay (Fig 7B) and densitometric analysis (Fig 7C) revealed that males have greater decline in the protein after hyperoxia exposure than females (p value<0.05 in WT males; p value<0.01 in *Cyp1a1*−/− males). Also, *Cyp1a1*−/− mice showed higher CYP1A2 protein levels compared to WT mice in room air. We performed RT-PCR with liver tissues at room air and after 24 h of hyperoxia exposure to look for the expression of CYP1A2 mRNA between WT and *Cyp1a1*−/− animals. The expression at 24 h (Fig 7D) was expressed as fold increase from WT male room air levels. In WT animals, females showed a significant induction in CYP1A2 mRNA expression at 24 h compared to room air levels ($p<0.05$), which is consistent with the higher activity and protein expression in females as described before. *Cyp1a1*−/− females showed higher baseline expression in room air compared to WT females.

3.6. Effect of hyperoxia on pulmonary CYP1A activity and protein levels

WT male mice had lower CYP1A1 activity at room air ($p<0.05$) and after exposure to hyperoxia for 72 h ($p<0.01$) when compared to WT females (Fig 8A). This sex specific difference was not seen in *Cyp1a2*−/− mice. Next, we determined the effect of hyperoxia on CYP1A1 protein expression in lung total protein fractions. Western blot assay of lung

protein (Fig 8B) and densitometric analysis (Fig 8C) of CYP1A1 protein expression in WT and *Cyp1a2*−/− mice at room air and after 48 h of hyperoxia exposure showed that there was a decline in CYP1A1 protein level in WT males, while *Cyp1a2*−/− males showed an increase in protein level ($p<0.05$). In the lung, RT-PCR for CYP1A1 expression showed no sex-specific differences (data not shown).

4. Discussion

In this study, we demonstrated the gender based dimorphic response in lung injury due to hyperoxia and its possible correlation with the differences in expression of CYP1A1 and CYP1A2 in male and female mice. WT male mice were more susceptible to hyperoxic injury compared to female mice. The WT male mice had more pulmonary edema, lung injury and neutrophil infiltration after hyperoxia exposure and this effect was either lost or reversed in *Cyp1a1* and *Cyp1a2*−/− mice.

The protective effect of CYP1A system against hyperoxic lung injury has been well documented. Induction of the CYP1A system, using agents like 3-MC and BNF (Mansour et al., 1988; Sinha et al., 2005) attenuates, while treatment with the CYP1A inhibitor 1-aminobenzotriazole (Moorthy et al., 2000) exacerbates lung injury in hyperoxic conditions. Since CYP1A1, but not CYP1A2 is expressed in lung, we only analyzed pulmonary expression of CYP1A1. Although CYP1A2 is mainly present in the liver and rarely expressed in the extra-hepatic tissues, *Cyp1a2*−/− mice are more susceptible to hyperoxic lung injury (unpublished results), and show evidence of increased ROS formation under hyperoxic conditions (Shertzer et al., 2004). This indicates that CYP1A2 may have extra-hepatic protective effects under these conditions. The induction of CYP1A by inducers like 3MC and BNF is thought to be mediated via the AhR pathway and we have observed that the AhR-deficient mice were refractory to CYP1A1 induction by hyperoxia and were more sensitive to lung injury than wild-type mice (Jiang et al., 2004).

Sex related differences in the constitutive expression of CYP1A have been observed in pigs (Kojima et al., 2010; 2008; Rasmussen et al., 2011). mRNA, protein and enzyme activities were higher in female and castrate male pigs suggesting down regulation of CYP1A expression by androgens. Testosterone treatment decreased the expression of CYP1A in both sexes of immature pigs (Kojima et al., 2008). Both estradiol and progesterone increased CYP1A1 activity and expression in mouse hepatoma Hepa1c1c7 cells (Lee et al., 1998). A decrease in AhR expression and Ah-responsiveness for the induction of CYP1A1 was observed in MCF-7 breast cancer cells grown in medium with low concentration of estradiol, which was reversed with estradiol supplementation (Spink et al., 2003). On the other hand testosterone has been shown to decrease CYP1A expression (Monostory et al., 2009).

To test whether CYP1A expression was different between male and female mice, which could correlate with the differences in lung injury, we conducted several experiments. Analysis of liver CYP1A2 mRNA by real time RT-PCR showed greater induction at 24 h of hyperoxia exposure in females compared to males. This indicates that exposure to hyperoxia leads to greater transcriptional activation of the *Cyp1a2* gene in the liver in female mice

compared to males, presumably via AhR-dependent mechanism. In the liver, we measured CYP1A2 activity using the MROD assay and, protein level using Western blot at baseline and after 72 h of hyperoxia exposure. WT males showed decreased hepatic CYP1A2 activity and a greater decline in the CYP1A2 activity and protein level after exposure to hyperoxia than females. Since CYP1A2 is protective against hyperoxic lung injury, this could explain the attenuation of lung injury in females compared to males. Our findings were contrary to Macak-Safranko et al (Mačak-Šafranko et al., 2011) where they exposed 1 month old CBA/Hr mice to 18 h of hyperoxia and measured CYP1A2 mRNA levels in male and female mice, and found that expression was decreased in females. The discrepancies in our observations may be due to different time points of measurement, different strain of mice etc. However, since the increase in mRNA expression at 24 h is accompanied with the relatively increased CYP1A2 activity in females in this study, and the fact that our unpublished data show the protective effects of CYP1A2 induction in hyperoxic lung injury, we conclude that there is a differential mechanistic relationship in males and females in regard to CYP1A2 expression and hyperoxic lung injury.

In the lungs, female WT mice had greater constitutional activity of CYP1A1, as measured by the EROD assay. After exposure to hyperoxia female mice have greater CYP1A1 activity and protein level when compared to males. The mRNA expression was not significant between the two groups. This may suggest a post-translational mechanism (e.g. protein stabilization) leading to increased enzyme activity and protein level in the females.

To further elucidate the role of CYP1A in the gender-based differences, we subjected male and female *Cyp1a1*−/− and *Cyp1a2*−/− mice of the same background to hyperoxia. Interestingly, in the knockout animals females lose the gender advantage and show greater lung injury than males. Pulmonary edema (as measured by change in lw/bw) was worse in females compared to their male counterparts. Lung histology showed more signs of injury in female mice compared to male mice. *Cyp1a2*−/− females showed greater neutrophil infiltration and TNF-α mRNA expression in their lungs compared to males. Induction of TNF-α in hyperoxic lung injury has been shown previously (Jensen et al., 1992). There have also been reports of sex-specific differences in its function (Kadokami et al., 2000) and its role in the repression of CYP1A1 and CYP1A2 under inflammatory conditions (Muntané-Relat et al., 1995). The increased lung injury and inflammation in *Cyp1a1*−/− and *Cyp1a2*−/− animals was similar to previously published research suggesting antioxidant activity of CYP1A2 (Shertzer et al., 2004). These findings suggest a gender-specific modulation of CYP1A in the setting of hyperoxic conditions in WT mice, which is lost in the *Cyp1a1*−/− and *Cyp1a2*−/− mice. Our results suggest an interaction between the CYP1A system and a female-specific mechanism involved in protection against hyperoxic lung injury. One possibility is the generation of 2-methoxyestradiol from 17- β estradiol by CYP1A1/A2 (hydroxylation) and subsequent methylation by catechol-*O*-methyl transferase (COMT) (Pribluda et al., 2000). The effects of 2-methoxyestardiol are independent of estrogen receptors alpha and beta (LaVallee et al., 2002). 2-methoxyestradiol has been shown to have anti-inflammatory effects (Duncan et al., 2012; Quezada et al., 2013; Stubelius et al., 2014). In the absence of CYP1A, in the knock-out mice, this protective metabolite is not produced in female mice compared to WT female mice, which may explain the reversal or loss of

better outcomes in *Cyp1a1*−/− and *Cyp1a2*−/− female mice. The reversal of sex-specific differences is more pronounced in the *Cyp1a2*−/− mice. This is an interesting finding as CYP1A2 is a liver-specific enzyme, suggesting the protective role of extra pulmonary organs like the liver in hyperoxic lung injury, especially in female mice.

In this study, we did not time our experiments with the estrous cycle in female mice, which could have led to some of the variability in our findings. However, consistent results across multiple experiments prove the validity of our findings. Other mechanisms have been proposed explaining the resistance of female mice to hyperoxia induced oxidative stress such as upregulated expression of heme oxygenase-1 and other cytochrome enzymes such as Cyp2a5 (Mačak-Šafranko et al., 2011). AhR also induces phase II enzymes such as NAD(P)H quinone reductase, glutathione S-transferase-α and aldehyde dehydrogenase, the beneficial effect of these enzymes cannot be excluded. Other antioxidant enzymes like superoxide dismutase could also have contributed towards the gender based differences observed (Park and Rho, 2002). Although hormones like estradiol (E2) and testosterone are a major contributor to sex-specific outcomes, there may be other mechanisms underlying these differences (Casimir et al., 2013). Sex-specific differences in apoptosis have been shown in neuronal ischemia, heart failure and renal ischemia. Divergent cell death pathway activation could be one of the reasons leading to this phenomenon (Lang and McCullough, 2008).

5. Conclusions

Our findings showing sex-specific differences in hyperoxic lung injury indicate a mechanistic role for CYP1A enzymes in the gender differences in oxygen injury. Since drugs can modulate CYP1A enzymes, we strongly believe that further research could lead to novel approaches for the prevention and/or treatment of acute lung injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by grants from National Institutes of Health [R01-HL-112516, R01-HL-087174, R01-ES-019689, and R01-ES-009132 to B.M., and R01-HL-088343 to XIC].

Abbreviations

AhR	aryl hydrocarbon receptor
AhRd mice	aryl hydrocarbon receptor dysfunctional mice
BNF	Beta-naphthoflavone
BPD	bronchopulmonary dysplasia
CYP	cytochrome P450
EROD	ethoxyresorufin-o-deethylase

3-MC	3- methylcholanthrene
MROD	methoxyresorufin-o-demethylase
O_2	Oxygen
ROS	reactive oxygen species
RDS	Respiratory Distress Syndrome

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Highlights

1. Cytochrome P450 (CYP)1A enzymes show gender-specific changes in hyperoxic lung injury.
2. WT female mice show higher CYP1A expression in hyperoxia compared to males.
3. The female advantage is lost or reversed in *Cyp1a1*−/− and *Cyp1a2*−/− mice.

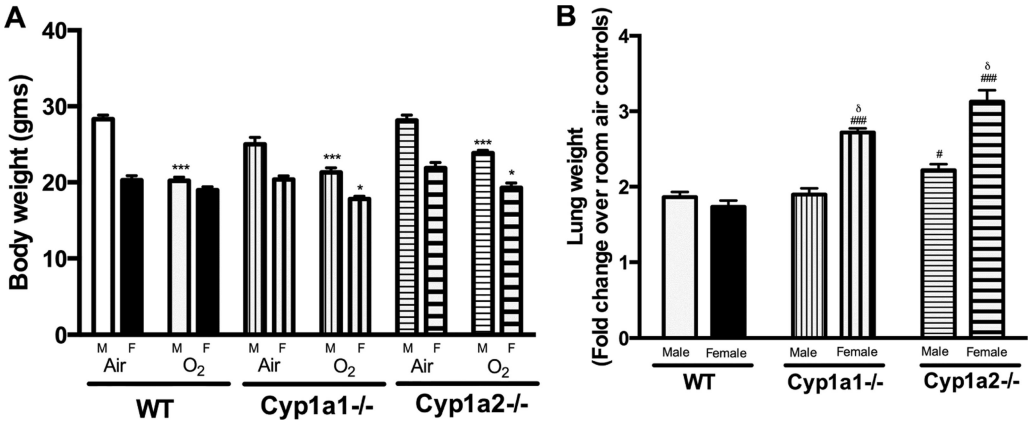

Figure 1.

Effect of hyperoxia (72 h) on lung weight and body weight: 1A: Effect of hyperoxia on body weights of WT, *Cyp1a1*−/− and *Cyp1a2*−/− mice. Values are means ± SEM from at least 5 individual animals. 1B: Fold change in lung weight following hyperoxia exposure in male and female WT, *Cyp1a1*−/− and *Cyp1a2*−/− mice. Values are means ± SEM from at least 3 individual animals. Significant differences between male and female mice are indicated by δ, $p < 0.05$, between WT and knock-out (*Cyp1a1*−/− or *Cyp1a2*−/−) mice within the same sex are indicated by #, $p < 0.05$ and ###, $p < 0.001$, and between room air and hyperoxia exposed mice are indicated by *, $p < 0.05$ and ***, $p < 0.001$.

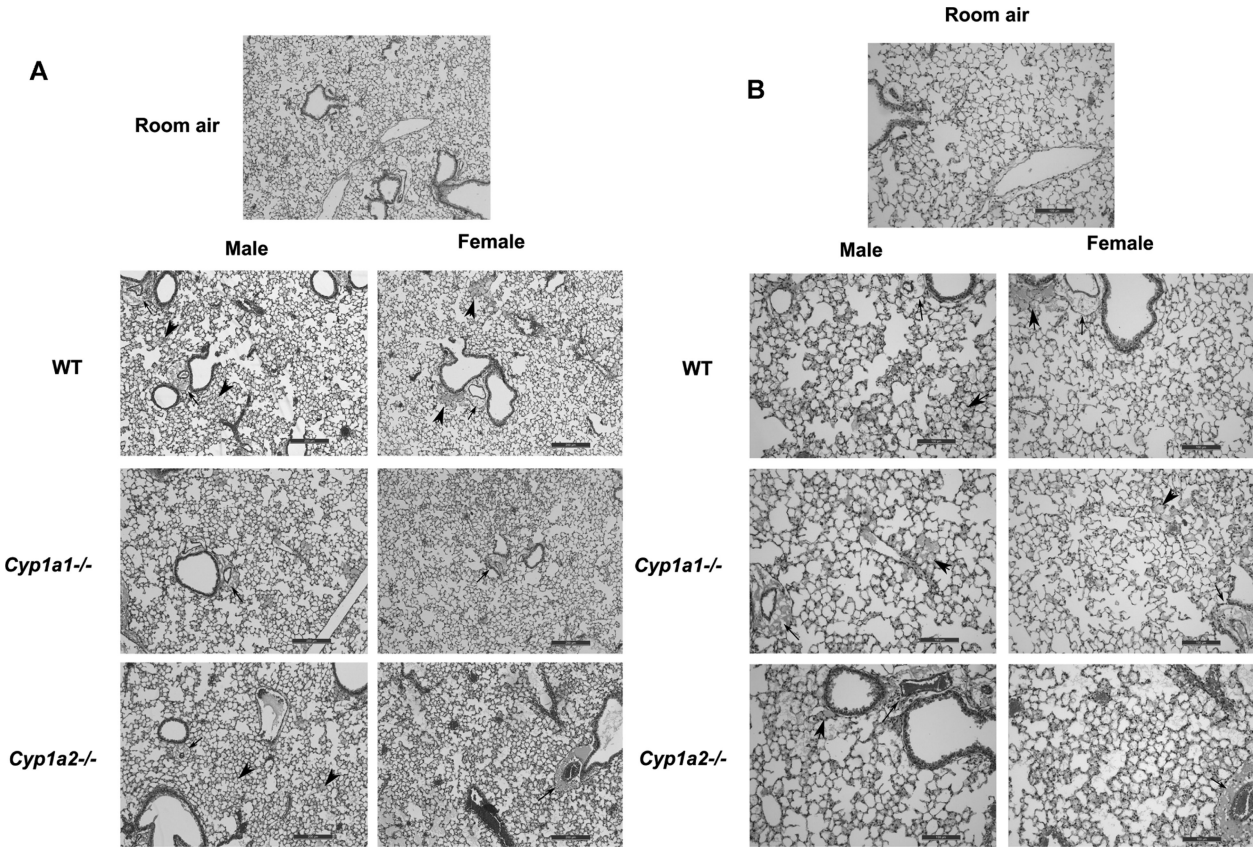

Figure 2. Effect of hyperoxia on lung histopathology

Representative hematoxylin eosin stained images from the lungs of WT and *Cyp1a1*−/− and *Cyp1a2*−/− mice (n=5 mice per group). WT, *Cyp1a1*−/− and *Cyp1a2*−/− mice were exposed to hyperoxia (72 h) at 4 x (2A) and 10x (2B) magnification of the lung fields. WT males show more perivascular, bronchiolar edema and alveolar hemorrhage when compared to female mice. *Cyp1a2*−/− females show greater signs of lung injury than males. Thick arrows point to areas of alveolar edema. Thin arrows point to regions showing perivascular edema.

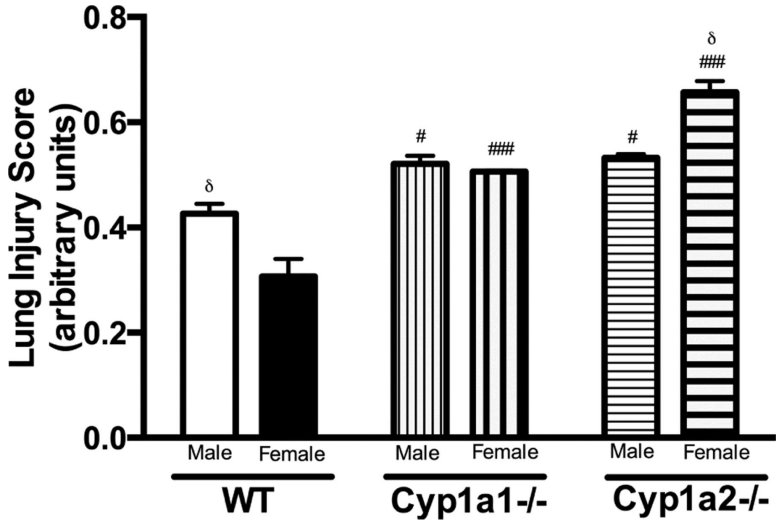

Figure 3. Lung injury in WT, *Cyp1a1*−/− and *Cyp1a2*−/− male and female mice

Lung injury scores in 20 random high-power fields (400× total magnification, n = 3 mice per group) in hematoxylin and eosin stained lung sections obtained from male and female mice of all three genotypes after exposure to hyperoxia (72 h). Significant differences between male and female mice are indicated by δ, $p < 0.05$, between WT and knock-out (*Cyp1a1*−/− or *Cyp1a2*−/−) mice within the same sex are indicated by #, $p < 0.05$ and ###, $p < 0.001$

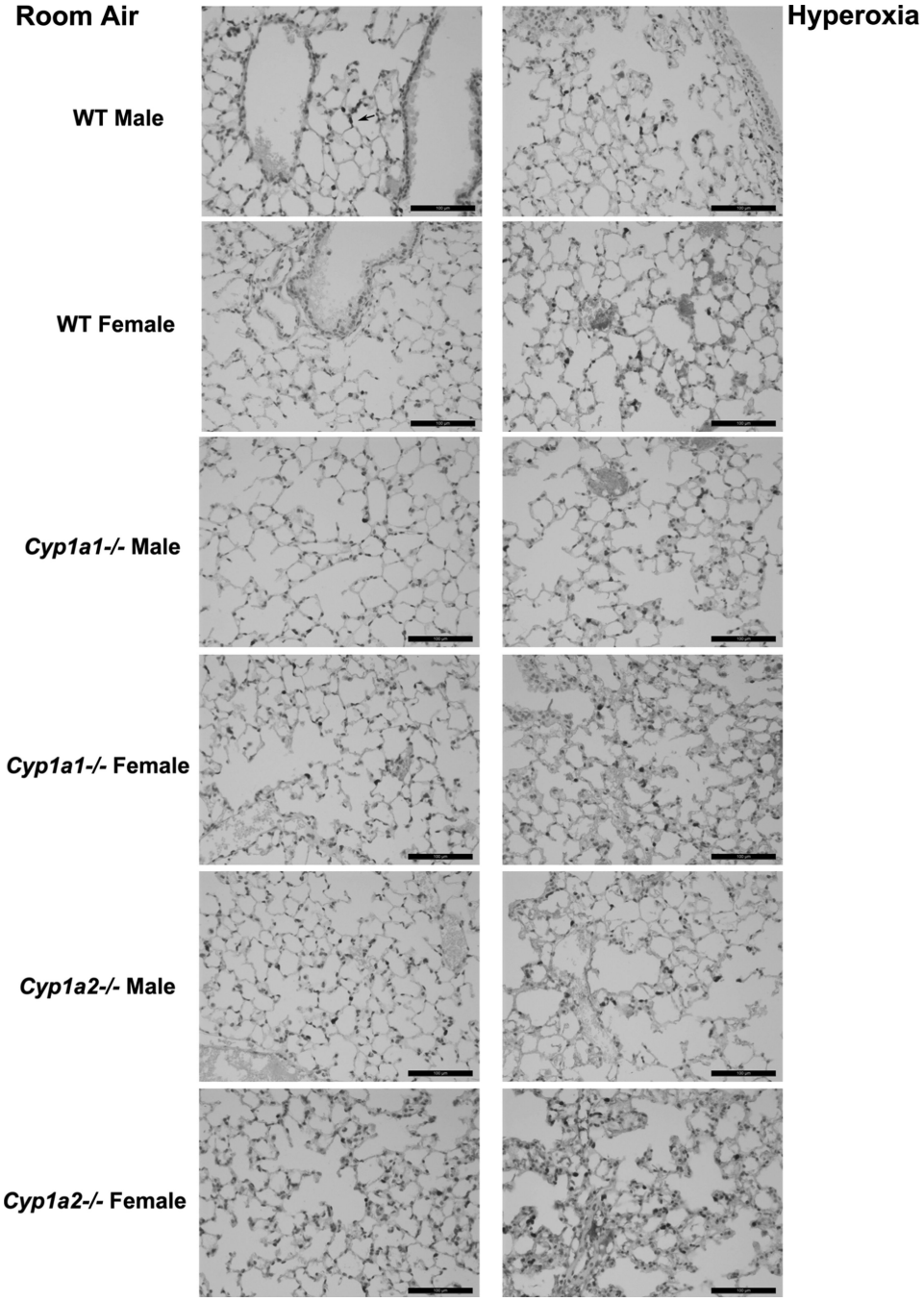

Figure 4.

Representative immunostained images for lung neutrophils. Hyperoxia-induced neutrophil recruitment was determined by immunohistochemistry with anti-neutrophil antibodies in WT, *Cyp1a1*−/− and *Cyp1a2*−/− mice (n=4 mice per group) at room air and after 72 h of hyperoxia. Arrow points to brown-staining neutrophils.

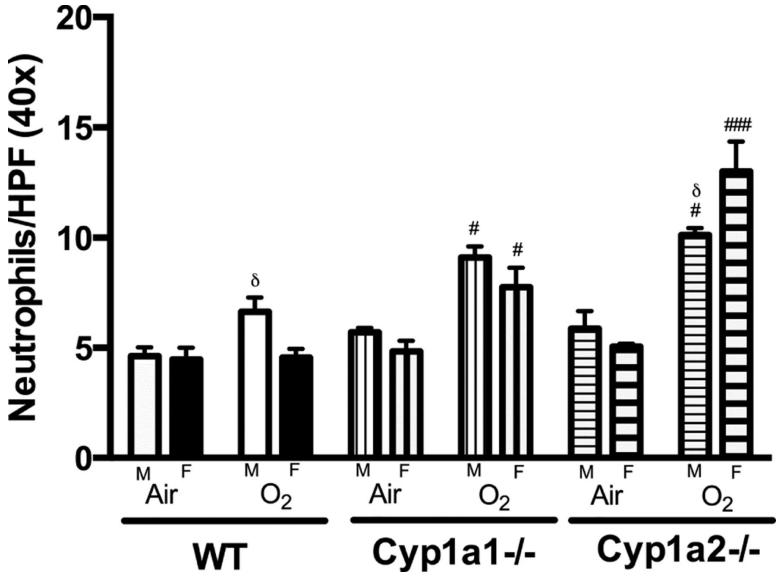

Figure 5.

Representative quantitative analysis of neutrophil infiltration in the lungs at room air and after exposure to 72 h of hyperoxia (FiO2>95%). Neutrophil count per high power (40x magnification) was done as described under materials and methods. Data represent means ± SEM from at least 3 individual animals in each group. Significant differences between male and female mice are indicated by δ, $p < 0.05$. Significant differences between WT and knock-out (*Cyp1a1*−/− or *Cyp1a2*−/− mice) within the same sex are indicated by #, $p < 0.05$ and ###, $p < 0.001$.

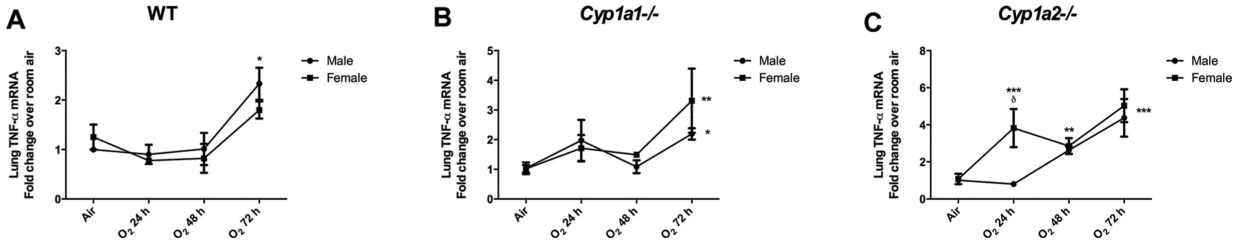

Figure 6.

Real time PCR analysis showing the fold increase in lung TNF- α mRNA expression in over baseline room air levels after 72 h of hyperoxia exposure. Values are means ± SEM from at least 3 individual animals. Figures 6A, 6B and 6C show the changes in male and female mice in WT, *Cyp1a1*−/− and *Cyp1a2*−/− mice. Significant differences between male and female mice are indicated by δ, $p < 0.05$. Significant differences form room air controls are indicated by *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

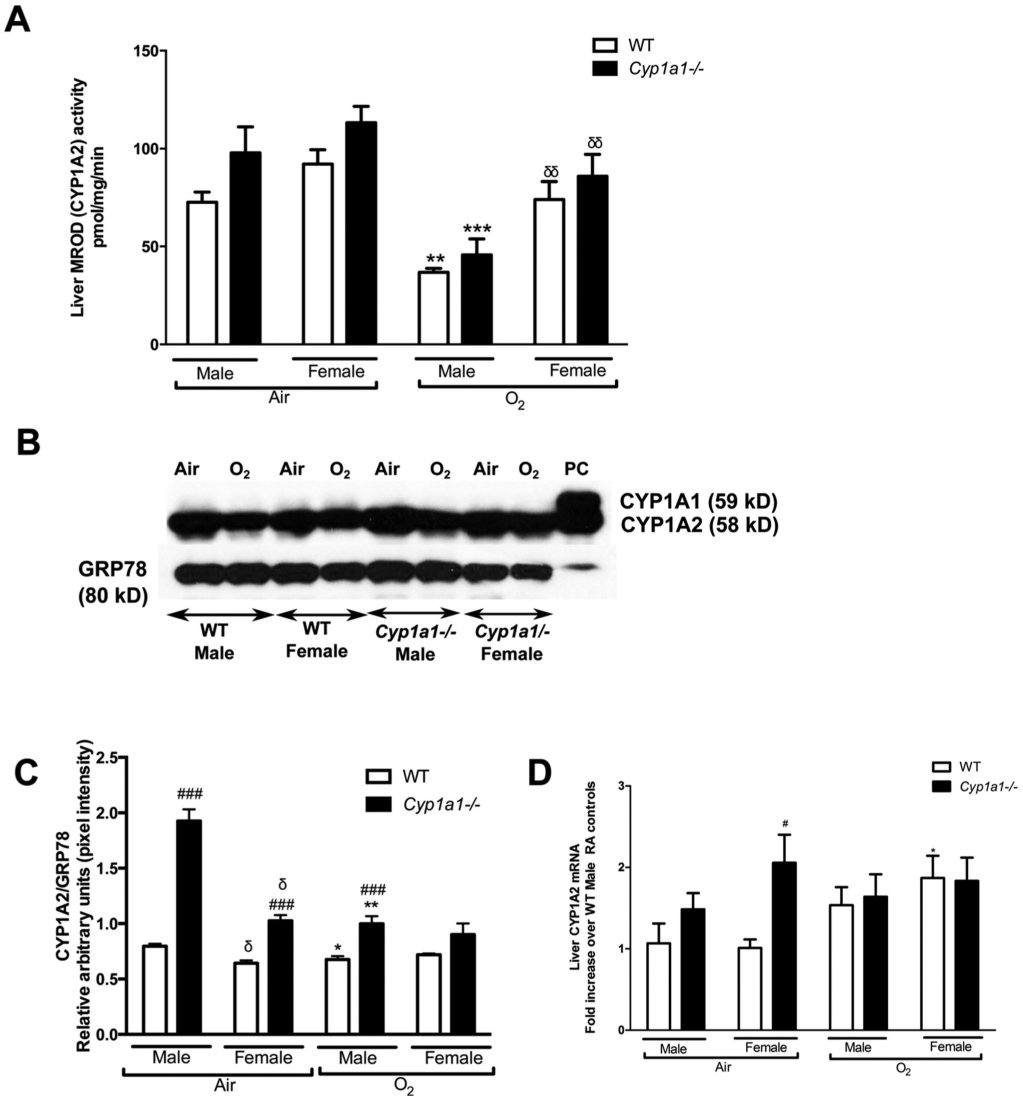

Figure 7.

Hepatic CYP1A activity and expression **7A:** MROD (CYP1A2) activity in the liver as measured by the MROD assay at room air (Air) and after 72 h of hyperoxia exposure (O_2). Data represent means±SEM from at least 4 individual animals in each group. **7B:** Representative western blot assays showing hepatic CYP1A2 (apoprotein) expression in liver microsomes isolated from WT and *Cyp1a1*−/− mice at room air (Air) and after 72 h of hyperoxia exposure (O_2). GRP-78 (glucose regulated protein; 78kDa) was used as the loading control. The positive control (labeled "PC") was 0.5 μg of liver microsomes from mice treated with 3-methylcholanthrene. **7C:** Densitometric analysis of hepatic CYP1A2 immunoblots. **7D:** Real-time RT-PCR analysis for CYP1A2 mRNA (Liver) in WT and *Cyp1a1*−/− mice. Values are means±SEM from at least 3 individual animals. Liver CYP1A2 mRNA expression at 24 h of hyperoxia exposure expressed as fold change over expression level in WT males at room air. Significant differences between male and female mice are indicated by δ, $p < 0.05$ and δδ, $p < 0.01$. Significant differences between WT and knock-out (*Cyp1a1*−/− mice) within the same sex are indicated by #, $p < 0.05$ and ###, $p<0.001$.

Significant differences form room air controls are indicated by *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

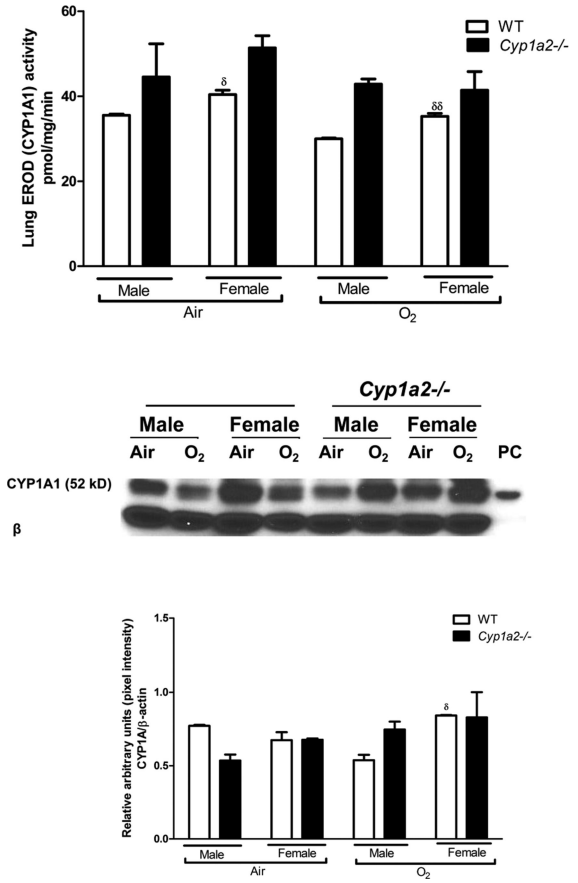

Figure 8.

Pulmonary CYP1A1 activity and western blot assay **8A:** CYP1A1 activity in the lung as measured by the EROD assay at room air (Air) and after 72 h of hyperoxia exposure (O_2). Data represent means±SEM from at least 4 individual animals in each group. Significant differences between male and female mice are indicated by δ, $p < 0.05$ and δδ, $p < 0.01$. **8B:** Whole lung proteins (20μg) were subjected to Western blotting using monoclonal antibodies raised against CYP1A1, as described in "Materials and methods". Whole lung proteins were isolated from WT and *Cyp1a2*−/− mice at room air (Air) and after 48 h of hyperoxia exposure (O_2). Under each sample lane is the corresponding β-actin blot to assess for protein loading. The positive control (labeled "PC") was 0.5 μg of liver microsomes from mice treated with 3-MC. **8C:** Densitometric analysis of pulmonary CYP1A1 immunoblots at room air and after 48 h of hyperoxia exposure (O_2). . Significant differences between male and female mice are indicated by δ, $p < 0.05$.