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Disruption of Cytochrome P4501A2 in mice leads to increased susceptibility to hyperoxic lung injury

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Abstract

Hyperoxia contributes to acute lung injury (ALI) in diseases such as acute respiratory distress syndrome (ARDS). Cytochrome P450 (CYP)1A enzymes have been implicated in hyperoxic lung injury, but the mechanistic role(s) of CYP1A2 in pulmonary injury is not known. We hypothesized that mice lacking the gene for *Cyp1a2* (which is predominantly expressed in the liver) will be more sensitive to lung injury and inflammation mediated by hyperoxia, and that CYP1A2 will play a protective role by attenuating lipid peroxidation and oxidative stress in the lung. Eight to ten week old WT (C57BL/6) or *Cyp1a2*(^{-/-}) mice were exposed to hyperoxia (>95% O₂) or maintained in room air for 24–72 h. Lung injury was assessed by determining the ratios of lung weight/body weight (LW/BW), and by histology. Extent of inflammation was determined by measuring the number of neutrophils in the lung as well as cytokine expression. The *Cyp1a2*(^{-/-}) mice under hyperoxic conditions showed increased LW/BW ratios, lung injury, neutrophil infiltration, IL-6 and TNF- α levels, and augmented lipid peroxidation, as evidenced by increased formation of malondialdehyde (MDA)- and 4-hydroxynonenal (4-HNE)-protein adducts, and pulmonary isofurans compared to those of WT mice. *In vitro* experiments showed that the F₂-isoprostane PGF₂- α is metabolized by CYP1A2 to a dinor metabolite, providing evidence for a catalytic role for CYP1A2 in the metabolism of F₂-isoprostanes. In summary, our results support

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the hypothesis that hepatic CYP1A2 plays a critical role in the attenuation against hyperoxic lung injury by decreasing lipid peroxidation and oxidative stress *in vivo*.

Keywords

Acute lung injury; CYP1A2; ARDS; oxidative stress

INTRODUCTION

Supplemental oxygen administration is often used in the clinical management of patients with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1]. In adults, ARDS represents acute respiratory failure that affects over 200,000 patients a year in the U.S. With an estimated mortality of 30–50%, ARDS accounts for about 3 million hospital days and contributes to significant morbidity [1,2]. Hyperoxia causes lung injury in experimental animals [3–6], and exacerbates ALI/ARDS in adults [7]. Reactive oxygen species (ROS) generated during hyperoxia exposure are known to play a role in pulmonary oxygen toxicity [8–12]. Hyperoxia exposure leads to a wide array of changes in the pulmonary transcriptome [13–15] including the transcription factor Nrf2 which in turn activates many anti-oxidant genes. Hyperoxia also activates many inflammatory mediators including cytokines [16]. The resulting acute lung injury can initiate and propagate a systemic inflammatory response leading to multi-organ dysfunction [17–19].

The cytochrome P450 (CYP) are involved in the metabolism of various exogenous and endogenous compounds [20,21]. CYP1A1 and CYP1A2 are induced by polycyclic aromatic hydrocarbons (PAHs) by arylhydrocarbon receptor (AHR)-dependent mechanisms [22,23]. While CYP1A1 [24] can be induced by PAHs in liver and many other tissues [25], CYP1A2 is predominantly expressed in the liver [25–27], and is not induced in extra-hepatic tissues even after treatment with PAHs [25].

Hyperoxia induces pulmonary CYP1A1 and hepatic CYP1A1/1A2 [8,28,29]. The underlying mechanisms are not clearly understood, although the AHR pathway has been proposed to mediate these effects [28,30,31]. P450 enzymes, including CYP1A1/1A2 have been shown to modulate pulmonary oxygen toxicity [28,29,32–35] but the mechanisms by which hepatic CYP1A enzymes modulate hyperoxic lung injury are not known. In human patients, the combination of severe liver dysfunction and ARDS carries a mortality of 100% in adults [36] and liver diseases such as cirrhosis are recognized as risk factors for the development of and mortality in ARDS [37,38]. The specific role of the liver-specific enzyme; CYP1A2 in the amelioration of lung injury due to hyperoxia has not been investigated. Hyperoxia adversely affects all cellular components including DNA, protein and lipid. Isoprostanes and isofurans are formed non-enzymatically by free radical-catalyzed peroxidation of arachidonic acid [39]. These are compounds are both markers and mediators of oxidative stress [40,41].

In this study, we tested the hypothesis that mice lacking the gene for *Cyp1a2*, which is predominantly expressed in the liver, will be more sensitive to lung injury and inflammation mediated by hyperoxia, and that hepatic CYP1A2 protects against lung injury in part by

mechanisms entailing decreases in the extent of lipid peroxidation, leading to a lowered level of oxidative injury.

MATERIALS AND METHODS

Chemicals

Tris, sucrose, NADPH, bovine serum albumin, ethoxyresorufin, methoxyresorufin, EDTA, and butylated hydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO). Buffer components for electrophoresis and Western blotting and Goat anti-mouse IgG conjugated with horseradish peroxidase were obtained from Bio-Rad laboratories (Hercules, CA). Rat anti Mouse Ly-6B.2 monoclonal antibody, clone 7/4 (Cat no: MCA771G) was purchased from AbD Serotec (Bio-Rad laboratories, Richmond CA), the primary monoclonal antibody to CYP1A1 was obtained from Dr. P.E. Thomas, Rutgers University. All real-time RT-PCR reagents were bought from Applied Biosystems (Foster City, CA).

Animals

The animal protocol was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (Protocol number: AN-907) and the study was conducted in strict accordance with federal guidelines for the humane care and use of laboratory animals by. C57BL/6J wild type (WT) mice were obtained from Charles River laboratories (Wilmington, DE) and creation of the *Cyp1a2*($-/-$) knockout mouse line, has been previously described [42]. These mice, which were on a mixed background (B6/SV129) were cross-bred into the C57BL/6J background by back-crossing for 12 generations, resulting in *Cyp1a2*($-/-$) on > 99% B6 background.

Animals were maintained at the Feigin Center animal facility of Baylor College of Medicine, Houston, TX. A 12 h day-night cycle was maintained throughout the study. Purified tap water and food (Purina Rodent Lab Chow No. 5001 from Purina Mills, Inc. Richmond, IN) were available *ad libitum*. WT or *Cyp1a2*($-/-$) mice both at eight to ten weeks old (3–6 mice per group) were maintained in room air or exposed to hyperoxia for 24, 48 or 72 h. For hyperoxia exposures, mice were placed in plexiglass chambers; oxygen was delivered through a humidified circuit at a flow rate of 5 L/min to achieve FiO_2 of >95%. Oxygen concentration was monitored continuously by means of in-line analyzers at the outport of the chambers [30]. In each group (room air, 24, 48 and 72 hr of hyperoxia exposure) 3 animals were used for lung histopathology and 6 animals were used for RNA and protein extraction and other studies.

Perfusion and tissue harvesting

At the end of each experiment, 9 mice from each group were sacrificed by exsanguination while under deep pentobarbital anesthesia. Lungs (n=3 mice) were perfused *in situ* with phosphate buffered saline (PBS), and used for subsequent analyses of CYP1A-dependent activities and protein contents. In the remaining three animals, the left lungs were inflated through an intra-tracheal catheter and fixed at constant pressure (20 cm H_2O) with zinc formalin, after which the lungs were embedded in paraffin for subsequent histological

analyses of lung injury [10]. The right lungs were snap-frozen at -80°C for subsequent RNA isolation.

Liver and lung microsome isolation

Liver microsomes were prepared by calcium chloride precipitation, as reported previously [43]. Lung microsomes were isolated by differential centrifugation, as published previously [44].

CYP1A1 and CYP1A2 enzyme assays

Ethoxyresorufin *O*-deethylase (EROD) (CYP1A1) activity in lung and liver microsomes was assayed according to the method of Pohl and Fouts [45], as reported earlier [30]. Methoxyresorufin *O*-demethylase (MROD) (CYP1A2) activity was assayed as described previously [30].

Western blotting

Liver and lung microsomes (20 μg protein) prepared from individual mice were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% acrylamide gels. The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by Western blotting using monoclonal antibodies to CYP1A1, which cross-reacts with CYP1A2 [10,30,31]. For loading controls, we used GRP78 (Glucose-regulated protein 78), a protein that is localized in the endoplasmic reticulum.

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

Total RNA (50 ng) from liver and lung of air-breathing or hyperoxic animals was subjected to one-step quantitative TaqMan RT-PCR. ABI PRISM 7700 Sequence Detection System was used for RT-PCR reactions, as previously described [31,46]. Serial dilutions of RNA were used to optimize and validate RT-PCR conditions for the *18S* gene and target genes chosen for this study. Each data point was repeated three times. Quantitative values were obtained from the threshold PCR cycle number (C_t) at which the increase in signal was associated with an exponential growth for PCR product becomes detectable. Relative mRNA levels for chosen target genes (i.e., *Cyp1a1*, *1a2*, *TNF- α* , *IL-6*) were normalized to *18S* RNA content. The primers for the target genes were obtained from Life Technologies (Carlsbad, CA); CYP1A1: Mm00487217_m1, TNF: Mm00443258_m1 and IL6: Mm00446190_m1, *18S*-Hs99999901_s1. For CYP1A2, the forward primer was: TCCTGGACTGACTCCCACAAC and the reverse primer was: GAACGCCATCTGTACCACTGAA. Relative expression levels of each target gene were calculated according to the equation, 2^{-C_t} , where $C_t = C_t \text{ target gene} - C_t \text{ 18S gene}$.

Lipid peroxidation and Inflammatory markers

Levels of F_2 -isoprostanes and isofurans in liver and lung homogenates were determined by GC-MS [39]. MDA- and 4-HNE-protein adducts in liver and lung tissue homogenates were analyzed by ELISA [47]. Serum and liver (tissue homogenates) were used to measure $\text{TNF-}\alpha$ levels by ELISA in WT and *Cyp1a2*($-/-$) mice.

Lung injury and Inflammation

Lung weight/body weight (LW/BW) ratios were calculated as an index of lung injury in animals whose lungs were not perfused for isolation of microsomes. This ratio was used as an index of hyperoxia-induced pulmonary edema formation. Lung histology was performed by H&E staining of lung sections, as described [10,30]. Assessment of lung injury in the histopathological lung sections was performed as described in the official American thoracic society workshop report on measurement of acute lung injury in experimental animals [48]. Twenty random high-power fields ($400 \times$ total magnification) were independently scored in a blinded fashion taking care that 50% of each field was occupied by lung alveoli. 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 lung injury score between zero and one. Neutrophil infiltration into lungs was determined by immunohistochemistry using the rat anti-mouse Ly-6B.2 monoclonal antibody, as reported [31]. Neutrophils were quantified by averaging the number of counts using at least 10 random high-power fields.

In vitro metabolism of $\text{PGF}_2\text{-}\alpha$

In order to determine the role of CYP1A2 in the metabolism of F_2 -isoprostanes, $\text{PGF}_2\text{-}\alpha$ (1 μM) was incubated with recombinant human CYP1A2 and NADP(H) cytochrome P450 oxidoreductase in PBS buffer, pH. 7.4 for 30 minutes, following which the reaction was stopped with chloroform:methanol (1:3), and the product(s) were extracted using the same solvent mixture. The chloroform:methanol fraction was evaporated to dryness, and spotted on silica gel thin-layer plates. The product, which was formed in the presence of CYP1A2 and P450 oxidoreductase, but not in the absence of oxidoreductase, was scrapped off from the plate, and eluted with ethylacetate, and evaporated to dryness, dissolved in acetonitrile, and identified by LC/MS/MS (ThermoFinnigan TSQ quantum quadrupole mass spectrometer) operating in negative ion mass mode and scanning from m/z 200 to m/z 500.

Statistical analysis

Data are expressed as means $\pm$ SEM. Two-way analyses of variance (ANOVA) (effect of genotype and treatment and interaction between the two main effects), was performed followed by modified t-tests, were used to assess significant differences arising from exposure to hyperoxia and room air, and between WT and *Cyp1a2*($-/-$) mice. *P*-values of < 0.05 were considered significant.

RESULTS

Cyp1a2($-/-$) mice show increased LW/BW ratios under hyperoxic conditions

WT and *Cyp1a2*($-/-$) mice exhibited significantly higher LW/BW ratios than room air controls after 48 and 72 h of hyperoxia ($>95\%$ FiO_2), with the most pronounced effect occurring in the *Cyp1a2*($-/-$) mice, which showed a doubling of LW/BW ratios compared to room air controls (Figure 1). When the LW/BW ratios were compared between *Cyp1a2*($-/-$) and WT mice, the former had significantly higher LW/BW ratios at 72 h ($\sim 42\%$) than those of the latter (Figure 1).

***Cyp1a2*(-/-) mice show greater histological lung injury than WT mice after hyperoxia**

Signs of lung injury in the form of alveolar and perivascular edema were observed in *Cyp1a2*^{-/-} mice at 48 h after hyperoxia exposure. At 72 h, both WT and *Cyp1a2*^{-/-} mice showed increased lung injury (alveolar and perivascular edema) compared to room air controls (Figure 2A). *Cyp1a2*^{-/-} mice showed much greater injury than WT mice after hyperoxia with severe alveolar and perivascular edema at the 72 h time point (Figure 2A). Figure 2B shows the assessment of lung injury entailing the official American Thoracic Society guidelines for the estimation of acute lung injury in experimental animals [48]. Increase in lung injury score in animals after exposure to hyperoxia for 72 h was seen in both WT and *Cyp1a2*^{-/-} mice. Also, the pulmonary injury score was significantly greater in *Cyp1a2*^{-/-} mice compared to WT mice after each time point of oxygen exposure (Figure 2B).

***Cyp1a2*(-/-) mice display increased number of neutrophils in the lungs after hyperoxia**

There was increased pulmonary neutrophil infiltration after hyperoxia exposure for 72 h in both WT and *Cyp1a2*^{-/-} mice (Figure 3A), with the *Cyp1a2*^{-/-} mice showing greater neutrophil recruitment than WT mice. Quantitative analyses (Figure 3B) showed about a 3-fold increase in number of neutrophils in the lungs of WT mice after 72 h of hyperoxia exposure compared to room air controls. In *Cyp1a2*^{-/-} mice, there was a marked increase in the number of neutrophils (~ 1.8-fold) after 24 h of hyperoxia over room air controls (Figure 3B). Interestingly, there was a significant decrease in neutrophil number after 48 h, followed by another increase (0.8-fold) after 72 h of hyperoxia. When neutrophil numbers were compared among *Cyp1a2*^{-/-} and WT mice, even in room air, the former showed a 2.75-fold increase over the latter. At the 24 and 72 h time points after hyperoxic exposure, the neutrophil recruitment was augmented by 1.8- and 0.8-fold compared to the corresponding WT counterparts, respectively (Figure 3B). The decrease in neutrophils at 48 h has been observed in acute lung injury models [49,50]. This may be due to neutrophil recruitment being mediated through different pathways at 24 and 72 h. In an ALI model, it was shown that, high levels of CXCL1, CXCL2 and CXCL5 mediate the early phase of neutrophil recruitment and increases in extracellular ATP mediate the late phase [51].

Effect of hyperoxia on pulmonary and hepatic CYP1A1 and CYP1A2 activities and apoprotein levels

There was significant induction in pulmonary ethoxyresorufin resorufin O-deethylase (EROD) CYP1A1 activity after 24 and 48 h of hyperoxia over room air levels in WT mice (Figure 4A). By 72 h, the levels returned to those seen in normoxia controls. *Cyp1a2*^{-/-} mice showed much higher (~60%) pulmonary EROD activities than WT mice under normoxic conditions (Figure 4A). After 48 and 72 h of hyperoxia, the *Cyp1a2*^{-/-} mice showed a significant decline in EROD activities compared to room air controls in the lung (Figure 4A). In liver, hyperoxia did not cause significant changes in EROD activities in WT mice, but there was significant decline after 72 h (Figure 4B). The *Cyp1a2*^{-/-} mice showed repressed EROD activities in room air as well as hyperoxic conditions (24 and 48 h) compared to those of WT mice (Figure 4B). In regard to liver methoxyresorufin O-

demethylase (MROD) (CYP1A2), hyperoxia caused induction of MROD activity in WT mice after 48 h, with activities declining after 72 h (Figure 4C).

In lungs, hyperoxia increased pulmonary CYP1A1 apoprotein contents, as determined by Western blotting, in WT mice (Figure 5A and B), with the induction declining at 72 h (Figure 5B). In *Cyp1a2*($-/-$) mice, under normoxia and after 24 h of hyperoxia exposure, pulmonary CYP1A1 protein expression was markedly enhanced compared to that in WT mice. There was a decline in the level of CYP1A1 (Figure 5B) after 24 h of oxygen exposure. CYP1A2 was absent in the lungs of WT or *Cyp1a2*($-/-$) mice (Figure 5A). In liver, CYP1A1 was not detectable in WT or *Cyp1a2*($-/-$) mice (Figure 5C). An increase in CYP1A2 protein expression after 48 h of hyperoxia in WT mice was noted, and the induction declined after 72 h (Figure 5C and D). CYP1A2 was not detectable in *Cyp1a2*($-/-$) mice as expected.

Effect of hyperoxia on hepatic CYP1A1/1A2 and pulmonary CYP1A1 gene expression

Hyperoxia for 24 h led to a significant increase in pulmonary CYP1A1 mRNA after 24 h, and declined to control after 48h in WT mice (Figure 6A). In *Cyp1a2*($-/-$) mice, CYP1A1 mRNA expression was augmented at all time points compared to the WT counterparts (Figure 6A). In the lung there was no expression of CYP1A2 in WT and *Cyp1a2*($-/-$) mice as shown in Figure 6A. In liver, hyperoxia did not cause any significant alterations in the expression of CYP1A1 mRNA in WT mice, but in *Cyp1a2*($-/-$) mice, there was a marked increase in the expression of CYP1A1 mRNA even in room air conditions, compared to that of WT mice (Figure 6B). In mice that were exposed to hyperoxia, there was a significant decline in CYP1A1 mRNA expression at 24 and 48 h, compared to room air controls (Figure 6B).

In regard to CYP1A2 gene expression in the liver, hyperoxia for 24 h resulted in a 35% increase in WT mice, but this induction declined to less than control levels (70%) by 48 h, and further declined to 10% of control by 72 h (Figure 6C). In *Cyp1a2*($-/-$) mice, there was no detectable expression of hepatic CYP1A2, which was significantly decreased compared to WT mice.

Effect of hyperoxia on cytokine levels

Hyperoxia for 24 h led to significant increase in TNF- α mRNA levels in WT and *Cyp1a2*($-/-$) mice, and this persisted through 72 h of hyperoxia (Figure 7A). Hyperoxia also elicited increased gene expression of IL-6 in both WT and *Cyp1a2*($-/-$) mice, with *Cyp1a2*($-/-$) mice displaying higher expression of IL-6 compared to WT at 72 h (Figure 7B). In serum of WT animals, TNF- α protein expression was significantly augmented after 24 h of hyperoxia, but the expression declined after 48–72 h (Figure 7C). *Cyp1a2*($-/-$) mice showed higher TNF- α expression at 72 h compared to WT mice. In liver, exposure to hyperoxia did not elicit any induction of TNF- α at 24–48 h in WT mice, but *Cyp1a2*($-/-$) mice showed increased TNF- α protein levels at 24 h compared to air-breathing controls (Figure 7D).

Effects of hyperoxic exposure on lipid peroxidation

The effect of hyperoxia on oxidative stress was determined by assessing the extent of lipid peroxidation in liver and lung by measuring protein adducts of MDA (Figure 8A and B) and 4-HNE (Figure 8C and D). Hyperoxia exposure did not alter MDA-protein adduct levels in liver of WT mice (Figure 8A). The MDA-protein adduct levels were higher in *Cyp1a2*($-/-$) mice compared to WT mice under normoxia, but after hyperoxia there was a decline in MDA-protein adducts in the *Cyp1a2*($-/-$) mice (Figure 8A). In lungs, there was a statistically significant increase (doubling) of MDA-protein adducts in *Cyp1a2*($-/-$) mice exposed to hyperoxia at 48 h (Figure 8B), and significantly higher than in WT mice at 48 and 72 h of hyperoxia exposure. In WT mice there was a modest increase in lung MDA levels, which was significant at 48 h.

In regard to 4-HNE-protein adducts, in WT mice hyperoxia exposure for 48–72 h resulted in a significant decrease in hepatic 4-HNE-protein adducts compared to room air controls (Figure 8C). On the other hand, *Cyp1a2*($-/-$) mice under hyperoxic conditions for 72 h showed augmentation of 4-HNE-protein adducts compared to room air (Figure 8C). When hepatic 4-HNE-protein adducts were compared among WT and *Cyp1a2*($-/-$) mice, hyperoxia for 48 and 72 h showed higher levels compared to the corresponding WT counterparts. In the lungs of WT and *Cyp1a2*($-/-$) mice, hyperoxia for 48 h augmented 4-HNE-protein adducts, compared to room air controls (Figure 8D).

In WT mice, hyperoxia for 24 h significantly increased levels of pulmonary F_2 -isoprostanes (Figure 8E); however, the levels of these compounds returned to room air levels at later time points. In *Cyp1a2*($-/-$) mice, the levels of F_2 -isoprostanes were not altered at any time point under hyperoxic conditions (Figure 8E). Pulmonary isofurans showed increases after 24 h of hyperoxia in WT mice (Figure 8F), but the levels returned to room air levels after 48–72 h of hyperoxia. In the *Cyp1a2*($-/-$) mice, the levels of isofurans were significantly increased at 48 h compared to the WT counterparts (Figure 8F). In liver, hyperoxia did not induce levels of isofurans at any of the time points, but *Cyp1a2*($-/-$) mice showed increased levels of isofurans at 48 h of hyperoxia, compared to the WT counterparts (Figure 8G).

In vitro metabolism of F_2 -isoprostanes

In order to determine the role of CYP1A2 in the metabolism of F_2 -isoprostanes, PGF $_{2\alpha}$, was incubated with purified CYP1A2 in the presence of NADPH cytochrome P450 oxidoreductase (CPR) and NADPH. The metabolites were extracted and analyzed by TLC as described in Materials and Methods in the online supplement. The major metabolite M1 was analyzed by LC-MS/MS. The unmetabolized isoprostane had a molecular ion peak at 353 (M-1) (not shown). The metabolite M1 composed exclusively of an ion with an m/z 253 (Figure 9A). The transformation of the parent compound of m/z 353 to a species of m/z 253 (loss of 100 amu) is suggestive of oxidative cleavage of the α (upper) side chain at the 5,6 double bond, as this would make the compound more hydrophobic (manifested as the larger R_f on TLC). Alternatively, this transformation may represent a 2,3 dinor metabolite where the carboxyl group has been converted to an aldehyde, with dehydration of the three hydroxyl groups. Incomplete dehydration would yield the m/z 271 species in negative ion mode (Figure 9B). This represents a novel P450-mediated reaction.

DISCUSSION

The primary objective of this investigation was to determine the functional role of hepatic CYP1A2 in hyperoxic lung injury using mice lacking the gene for CYP1A2, which is predominantly expressed in the liver, and is absent in the lung. We found increased LW/BW ratios, lung injury (based on histopathology), and inflammation (increased neutrophil recruitment) in *Cyp1a2*($-/-$) mice exposed to hyperoxia, compared to similarly exposed WT mice, suggesting that liver CYP1A2 plays a protective role against lung injury and inflammation. The fact that the number of neutrophils in *Cyp1a2*($-/-$) mice was higher compared to WT mice even under normoxia, supported the hypothesis that endogenous hepatic CYP1A2 plays a protective role against lung inflammation in WT mice.

Induction of pulmonary EROD activity after 48 h of hyperoxia in WT mice probably reflected CYP1A1 induction by hyperoxia, because EROD activity is largely catalyzed by CYP1A1 [8,30]. The decline in EROD induction after 72 h of hyperoxia was similar to previous findings in adult rat [8,30,33] and mouse models [31] showing a decline in CYP1A1 expression after prolonged hyperoxia, coinciding with respiratory distress and lung injury. The augmentation of pulmonary EROD activities in *Cyp1a2*($-/-$) mice compared to WT mice could be explained by CYP1A2 metabolizing endogenous ligands of the AHR in the WT mice; therefore, in the *Cyp1a2*($-/-$) mice, the AHR ligands would accumulate, resulting in CYP1A1 induction.

In liver, the decrease in EROD activity after 72 h in WT was in agreement with studies reported previously in rats [8]. The augmentation of MROD expression in the livers of WT animals exposed to hyperoxia reflected CYP1A2 induction. The observation that modulation of EROD and MROD activities by hyperoxia in liver and lung correlated overall with apoprotein expression of CYP1A1 and CYP1A2 supported the theory that the hyperoxia effects were not due to enzyme stabilization.

The augmentation of pulmonary CYP1A1 mRNA in the *Cyp1a2*($-/-$) in room air, compared to that in WT mice may have been due to accumulation of AHR ligands that are CYP1A2 substrates. The induction of pulmonary CYP1A1 mRNA by hyperoxia for 24 h in the *Cyp1a2*($-/-$) was also possibly mediated by AHR-mediated mechanisms, based on previous findings [31] in which hyperoxia did not elicit pulmonary CYP1A1 expression in AHR-deficient mice. The induction of hepatic CYP1A2 mRNA expression by hyperoxia for 24 h, and the subsequent decline after 48–72 h was consistent with previous findings in the rat model [8,30]. The marked induction of hepatic CYP1A1 mRNA in air-breathing *Cyp1a2* $-/-$ mice was probably due to accumulation of AHR ligands that are cleared by CYP1A2 in the WT mice.

The enhancement of *TNF- α* and *IL-6* mRNA levels in lungs of WT mice after hyperoxia for 24–72 h suggested that hyperoxia caused an inflammatory response. These results were in agreement with previous studies showing that hyperoxia leads to increased *TNF- α* levels in the lung [52–55]. The role of cytokines in hyperoxic acute lung injury has been extensively studied [56] and other mediators such as *IL-1*, *IL-8*, *IFN- γ* , *MIP-2* and *MCP-1* play a role in this disease process.

That hyperoxic exposures led to increased levels of serum TNF α levels, a phenomenon that was even more pronounced in *Cyp1a2*($-/-$) mice suggested that hyperoxia elicits a systemic inflammatory response, and that in the absence of CYP1A2, this response is magnified. The present results also showed increased levels of TNF- α in livers of *Cyp1a2*($-/-$), supporting the hypothesis that in the normal mouse, hepatic CYP1A2 plays a protective role against inflammation. Systemic inflammation in response to lung injury has been observed by other researchers. Acute lung injury impairs the integrity of the epithelial and endothelial barrier and the alveolar cytokines and other pro-inflammatory mediators generated in the lung can leak into the vascular system. This can lead to a systemic inflammatory response [19,57]. Tutor et al demonstrated increase in systemic levels of TNF- α in acute lung injury [18]. St. John et al showed that neutrophils played a major role in the systemic inflammatory effects in an acid-aspiration model of acute lung injury [58]. Jobe et al have reported increase in hepatic markers of inflammation in preterm models of lung injury [17,59].

Increased levels of hepatic MDA-protein adducts in *Cyp1a2*($-/-$) mice under normoxia, compared to WT mice suggest that hepatic CYP1A2 metabolizes precursors of compounds that contribute to lipid peroxidation. The augmentation of pulmonary MDA-protein adducts in *Cyp1a2*($-/-$) mice after 48 h of hyperoxia further suggests that MDA-protein adducts contributed to hyperoxic lung injury and inflammation. The enhancement of 4-HNE adducts in livers of *Cyp1a2*($-/-$) mice exposed to hyperoxia indicated the protective role of liver CYP1A2 against oxygen-mediated lipid peroxidation.

The increased pulmonary F₂-isoprostane levels after hyperoxia in WT mice after 24 h, but not after 48 or 72 h, suggests that formation of lipid hydroperoxides precedes lung injury. The elevation in the levels of pulmonary F₂-isoprostanes and isofurans in rabbits following exposure to hyperoxia has been shown before [39], and has been attributed to be due to oxidative stress *in vivo*. The induction of CYP1A2 activity at the similar time point followed by a decrease in isofuran and F₂-isoprostane levels suggest a possible role for CYP1A2 in the detoxification of these lipid peroxidation products. *Cyp1a2*($-/-$) mice show significantly increased isofuran levels after 48h, compared with WT, suggesting that CYP1A2 metabolizes isofurans in the normal animal. Increased F₂-isoprostane levels have been correlated with lung injury in neonates many of whom receive supplementary oxygen therapy during their treatment course [60]. F₂-isoprostanes in tracheal aspirates were increased in the lungs of premature babies receiving oxygen and mechanical ventilation treated with the CYP inhibitor cimetidine, consistent with the hypothesis that CYP enzymes may attenuate the risk of oxidative injury [61].

In order to determine the role of CYP1A2 in the detoxification of lipid hydroperoxides, we conducted *in vitro* experiments with recombinant human CYP1A2, and the present results showing the formation of a dinor metabolite from PGF₂- α (Figure 9B) provide direct evidence that F₂-isoprostanes are endogenous substrates for CYP1A2. To our knowledge, this is the first report demonstrating a functional role for CYP1A2 in F₂-isoprostane metabolism.

It is not clear as to how F₂-isoprostanes/isofurans would contribute to lung injury. Seto et al [62] showed that the E-ring isoprostane, 15-E₂-isOP through an EP₄ receptor coupled to

adenylate cyclase and soluble guanylate cyclase, activates a transepithelial Cl conductance in bovine airway epithelium. This could account for the increased mucus production observed in lung injury models. Another possibility is that F₂-isoprostanes/isofurans might contribute to pulmonary vessel injury, which could in turn lead to lung injury, and this idea stems from the finding that these molecules cause constriction of isolated vessels, and could lead to pulmonary hypertension *in vivo*. F₂-isoprostanes/isofurans may also contribute to lung injury by enhancing production of pro-inflammatory cytokines by smooth muscle, endothelial and inflammatory cells. The fact that isoprostanes are elevated in patients with acute lung injury (ALI) [63] and in experimental animals exposed to hyperoxia [39,64] lend credence to the theory that these molecules play a causative role in hyperoxic lung injury. Involvement of other reactive compounds such as eicosanoids and leukotrienes [65], or phosphatidyl serine and cardiolipin [66] in hyperoxic lung injury cannot be excluded in this study. Only a limited number of inflammatory markers were investigated in this study and sex-specific differences were not looked into. A more detailed analysis of the transcriptome between WT and knock-out animals and sex-specific differences are being currently studied in our lab.

CONCLUSIONS

In summary, in this study, we have documented the protective effects of mammalian hepatic CYP1A2 against hyperoxic lung injury (Figure 10), revealing a critical role for extra-pulmonary organs such as liver in the protection against lung injury. We postulate that hyperoxia elevates ROS-mediated lipid hydroperoxides levels in lung and liver. In the lung, these substances may cause injury via cytokine activation, leading to inflammatory cell recruitment, and eventual injury and cell death. These may also enter the systemic circulation and reach the liver where they are detoxified by CYP1A2. In the absence of CYP1A2 (in *Cyp1a2*($-/-$) mouse), these compounds would magnify the inflammatory response to hyperoxia exposure, resulting in activation of pro-inflammatory cytokines (e.g., TNF- α and IL-6), which could result in greater lung injury. Another explanation could be that the F₂-isoprostanes/isofurans that are formed in the liver directly by hyperoxia may undergo detoxification by CYP1A2 to non-toxic metabolites. In the absence of CYP1A2, these compounds would accumulate, and may be transported to the systemic circulation, and this could in turn lead to cytokine activation and increased lung injury (Figure 10). In fact, the increased lung injury in *Cyp1a2*($-/-$) mice may entail a combination of both of these mechanisms. Thus, this study highlights the protective role of liver-specific CYP1A2 in metabolizing potential mediators of acute lung injury and provides a mechanism by which liver function could play a major role in determining survival in patients with ARDS. Further studies on the functional role of CYP1A2 in oxygen-mediated lung injury could lead to novel treatment/preventive strategies for ALI/ARDS.

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ABBREVIATIONS

4-HNE	4-hydroxynonenal
ARDS	Acute Respiratory Distress Syndrome
ALI	Acute Lung Injury
AHR	Aryl hydrocarbon receptor
CYP	Cytochrome P450
ELISA	Enzyme linked immunosorbent assay
EROD	Ethoxyresorufin <i>O</i> -deethylase
GC-MS	Gas Chromatography-Mass Spectrometry
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
LW/BW	Lung weight/Body weight
MDA	Malondialdehyde
MROD	Methoxyresorufin <i>O</i> -demethylase
ROS	Reactive Oxygen Species
TLC	Thin Layer Chromatography
WT	Wild-type

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HIGHLIGHTS

- CYP1A2 attenuates hyperoxic lung injury and inflammation.
- CYP1A2 decreases lipid peroxidation and oxidative stress.
- Extra-pulmonary organs such as liver may be protective against acute lung injury.

Figure 1.

Effect of hyperoxia on LW/BW ratios in WT and *Cyp1a2*(^{-/-}) mice. Representative quantitative analysis of the effects of hyperoxia exposure on LW/BW, a measure of pulmonary edema of WT and *Cyp1a2*(^{-/-}) mice. Values are means $\pm$ S.E.M. from six individual animals. Significant differences between room air and hyperoxia within genotype are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Significant differences between corresponding WT and *Cyp1a2*(^{-/-}) groups are indicated by ### $P < 0.001$. Main effects of genotype and treatment and interaction between the two main effects are also shown.

Figure 2.

H & E staining of lung histology and quantitation of lung injury. (A) *Cyp1a2*^(-/-) mice show greater hyperoxia-induced lung edema and perivascular and alveolar injury, compared with that in WT mice. Representative hematoxylin/eosin-stained images from WT vs. *Cyp1a2*^(-/-) lungs (n = 5 mice per group) are shown in room air and after 24, 48, and 72 h of hyperoxia exposure. Arrows and arrowheads point to perivascular and alveolar areas, respectively. (B) Lung injury scores in 20 random high-power fields (n = 3 mice per group) in hematoxylin and eosin stained lung sections obtained from WT and *Cyp1a2*^(-/-) mice at room air and after exposure to hyperoxia (24, 48 and 72 h). Significant differences between room air and hyperoxia within genotype are indicated by ** $P < 0.01$ and *** $P < 0.001$. Significant differences between corresponding WT and *Cyp1a2*^(-/-) groups are indicated by # $P < 0.05$ and ### $P < 0.001$. Main effects of genotype and treatment and interaction between the two main effects are also shown.

Figure 3.

Immunohistochemistry and quantitation of pulmonary neutrophil recruitment. (A) Representative immuno-stained images for lung neutrophils. Hyperoxia-induced neutrophil recruitment was determined by immunohistochemistry with anti-neutrophil antibodies in WT and *Cyp1a2*(^{-/-}) mice (n=5 mice per group) in room air and after 24, 48, and 72 h of hyperoxia. Arrows point to brown-staining neutrophils. (B) Quantitative analyses showing number of neutrophils per high-power field. Representative quantitative analysis of the hyperoxia effects on neutrophil recruitment in lungs of WT vs. *Cyp1a2*(^{-/-}) mice. Values are means $\pm$ S.E.M. from at 3 individual animals. Significant differences between room air and hyperoxia within genotype are indicated by * P < 0.05 and *** P < 0.001. Significant differences between room air and hyperoxia within genotype are indicated by * P < 0.05, ** P < 0.01 and *** P < 0.001. Significant differences between corresponding WT and *Cyp1a2*(^{-/-}) groups are indicated by # P < 0.05, ## P < 0.01 and ### P < 0.001.

Figure 4.

Representative quantitative analysis of EROD (CYP1A1 activity) in liver and lung and MROD (CYP1A2 activity) in liver of WT and *Cyp1a2*^{-/-} mice exposed to room air or hyperoxia for 24, 48 and 72 h. Pulmonary EROD (A), hepatic EROD (B), and hepatic MROD (C) activities were measured by spectrofluorometry. Values represent means $\pm$ S.E.M. from 3 individual animals. Significant differences between room air and hyperoxia within genotype are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Significant differences between corresponding WT and *Cyp1a2*^{-/-} groups are indicated by ### $P < 0.01$ and #### $P < 0.001$. Main effects of genotype and treatment and interaction between the two main effects are also shown.

Figure 5.

Effect of hyperoxia on CYP1A1/1A2 protein expression in mice. Representative Western immunoblots and densitometric analysis showing pulmonary (A and B) CYP1A1 and hepatic (C and D) CYP1A2 protein amounts in microsomes isolated from WT and *Cyp1a2*^{-/-} mice in room air (Air) and after 24, 48 and 72 h of hyperoxia. The positive control (labeled “PC”) was 0.5 µg of liver microsomes from 3-methylcholanthrene-treated WT mice. Under each sample lane is the corresponding GRP-78 (Glucose-regulated protein) blot to assess for protein loading. Significant differences between room air and hyperoxia within genotype are indicated by * $P < 0.05$ and *** $P < 0.001$. Significant differences between corresponding WT and *Cyp1a2*^{-/-} groups are indicated by ### $P < 0.001$. Main effects of genotype and treatment and interaction between the two main effects are also shown.

Figure 6.

Effect of hyperoxia on CYP1A1/1A2 mRNA expression in mice. Real time RT-PCR analysis showing effects of hyperoxia on hepatic CYP1A1 (A), pulmonary CYP1A1/A2 (B) and hepatic CYP1A2 (C) mRNA expression in WT vs. *Cyp1a2*(^{-/-}) mice. Values are means $\pm$ S.E.M. from 3 individual animals. Significant differences between room air and hyperoxia within genotype are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Significant differences between corresponding WT and *Cyp1a2*(^{-/-}) groups are indicated by ## $P < 0.01$ and ### $P < 0.001$. Main effects of genotype and treatment and interaction between the two main effects are also shown.

Figure 7.

Effect of hyperoxia on inflammatory markers in WT and *Cyp1a2*(^{-/-}) mice. Cytokine levels in lungs (RT-PCR), serum and liver (ELISA) showing effects of hyperoxia on pulmonary (A & B) TNF- α and IL-6 mRNA levels, serum (C) and liver (D) TNF- α levels measured by ELISA in WT and *Cyp1a2*(^{-/-}) mice. Values denote means $\pm$ S.E.M. from 3 individual animals. Significant differences between room air and hyperoxia within genotype are indicated by ** $P < 0.01$ and *** $P < 0.001$. Significant differences between corresponding WT and *Cyp1a2*(^{-/-}) groups are indicated by # $P < 0.05$ and ## $P < 0.01$. Main effects of genotype and treatment and interaction between the two main effects are also shown.

Figure 8.

Assessment of lipid peroxidation. Representative quantitative analysis of hyperoxia effects on lipid peroxidation in WT and *Cyp1a2*^{-/-} mice in liver (A and C) and lung (B and D) as measured by levels of malondialdehyde and 4-HNE- protein adducts. Also represented are levels of lung F₂-isoprostanes (E), lung isofurans (F) and liver isofurans (G). Values denote means $\pm$ S.E.M. from 3 individual animals. Significant differences between room air and hyperoxia within genotype are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Significant differences between corresponding WT and *Cyp1a2*^{-/-} groups are indicated by # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$. Main effects of genotype and treatment and interaction between the two main effects are also shown.

Figure 9.

In vitro metabolism of PGF₂-α. PGF₂-α (1 μM) was incubated with recombinant human CYP1A2 and NADP(H) cytochrome P450 oxidoreductase in PBS buffer, pH. 7.4 for 30 minutes, following which the reaction was stopped with chloroform:methanol (1:3), and the product(s) were extracted using the same solvent mixture. The chloroform:methanol fraction was evaporated to dryness, and spotted on silica gel thin-layer plates. The product, which was formed in the presence of CYP1A2 and NADP(H) P450 oxidoreductase, but not in the absence of oxidoreductase, was scrapped off from the plate, and eluted with ethylacetate, and evaporated to dryness, dissolved in acetonitrile, and identified by LC/MS/MS (ThermoFinnigan TSQ quantum quadrupole mass spectrometer) (A) operating in negative

ion mass mode and scanning from m/z 200 to m/z 500. B. Shows the possible formation of dinor metabolite after catalysis by CYP1A2.

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Figure 10.

Schematic of the lung protective role of hepatic CYP1A2 in hyperoxic lung injury. Hyperoxia elevates ROS-mediated lipid hydroperoxides (e.g., F₂-isoprostanes/isofurans) levels in lung, which then enter the systemic circulation and reach the liver where they are detoxified by CYP1A2. In the absence of CYP1A2 (in *Cyp1a2*^{-/-} mouse), these compounds would magnify the inflammatory response to hyperoxia exposure, resulting in activation of pro-inflammatory cytokines (e.g., TNF- α and IL-6), which could result in greater lung injury. Alternatively, the F₂-isoprostanes/isofurans that are formed in the liver directly by hyperoxia may undergo detoxification by CYP1A2 to non-toxic metabolites. In the absence of CYP1A2, these compounds would accumulate, and may be transported to the systemic circulation, and this could in turn lead to cytokine activation and increased lung injury.