Biochemical and Biophysical Research Communications 407 (2011) 79–85

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Augmented oxygen-mediated transcriptional activation of cytochrome P450 (CYP)1A expression and increased susceptibilities to hyperoxic lung injury in transgenic mice carrying the human CYP1A1 or mouse 1A2 promoter *in vivo* ☆

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ARTICLE INFO

Article history:
Received 22 February 2011
Available online 6 March 2011

Keywords:
Cytochrome P4501A1
CYP1A2
Hyperoxia
Promoter
Luciferase reporter
In vivo
Mice
Gene regulation
Transgenic mice
Lung injury

ABSTRACT

Supplemental oxygen administration is frequently administered to pre-term and term infants having pulmonary insufficiency. However, hyperoxia contributes to the development of bronchopulmonary dysplasia (BPD) in premature infants. Cytochrome P450 (CYP)A enzymes have been implicated in hyperoxic lung injury. In this study, we tested the hypothesis that hyperoxia induces CYP1A1 and 1A2 enzymes by transcriptional activation of the corresponding promoters *in vivo*, and transgenic mice expressing the human CYP1A1 or the mouse 1A2 promoter would be more susceptible to hyperoxic lung injury than wild type (WT) mice. Adult WT (CD-1) (12 week-old) mice, transgenic mice carrying a 10 kb human CYP1A1 promoter and the luciferase (luc) reporter gene (CYP1A1-luc), or mice expressing the mouse CYP1A2 promoter (CYP1A2-luc) were maintained in room air or exposed to hyperoxia for 24–72 h. Hyperoxia exposure of CYP1A1-luc mice for 24 and 48 h resulted in 2.5- and 1.25-fold increases, respectively, in signal intensities, compared to room air controls. By 72 h, the induction had declined to control levels. CYP1A2-luc mice also showed enhanced luc expression after 24–48 h, albeit to a lesser extent than those expressing the CYP1A1 promoter. Also, these mice showed decreased levels of endogenous CYP1A1 and 1A2 expression after prolonged hyperoxia, and were also more susceptible to lung injury than similarly exposed WT mice, with CYP1A2-luc mice showing the greatest injury. Our results support the hypothesis that hyperoxia induces CYP1A enzymes by transcriptional activation of its corresponding promoters, and that decreased endogenous expression of these enzymes contribute to the increased susceptibilities to hyperoxic lung injury in the transgenic animals. In summary, this is the first report providing direct evidence of hyperoxia-mediated induction of CYP1A1 and CYP1A2 expression *in vivo* by mechanisms entailing transcriptional activation of the corresponding promoters, a phenomenon that has implications for hyperoxic lung injury, as well as other pathologies caused by oxidative stress.

1. Introduction

Supplemental oxygen is frequently used in the treatment of pulmonary insufficiency in pre-term and term neonates [1,2]. Considerable evidence links oxygen exposure to the development of neonatal diseases such as bronchopulmonary dysplasia (BPD), a major cause of morbidity and mortality in premature infants [1–6]. Hyperoxia causes lung injury in animal models [2,7,8], and reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide are most likely to play important roles in mediating lung injury [9–13].

The cytochrome P450 (CYP) system of enzymes is a superfamily of heme-containing proteins that is involved in the metabolism of numerous exogenous and endogenous compounds [14]. The CYP1A family comprises of two proteins, i.e. CYP1A1 and 1A2, which are classically induced by planar aromatic hydrocarbons, such as benzo[α]pyrene found in cigarette smoke, or 3-methylcholanthrene [15]. Previous work in rodent models has shown that hyperoxia induces CYP1A enzymes [9,12,13,16–18], but the mechanisms involved are not clearly understood. In the classic model of CYP1A1 induction, the ligand binds to the arylhydrocarbon receptor (AHR),

Abbreviations: BPD, bronchopulmonary dysplasia; CYP, cytochrome P450; AHR, Ah receptor; EROD, ethoxyresorufin *O*-demethylase; MROD, methoxyresorufin *O*-demehylase; luc, luciferase; ANOVA, analyses of variance; AHREs, Ah response elements; ARNT, Ah receptor nuclear translocator; RT-PCR, reverse transcriptase-polymerase chain reaction; WT, wild type.

☆ This work was supported in part by National Institute of Health Grants RO1HL070921, R01HL087174, and RO1ES019689 to B.M.

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doi:10.1016/j.bbrc.2011.02.113

a cytosolic protein, and causes translocation of the AHR–ligand complex into the nucleus. There, it forms a heterodimer with the aryl-hydrocarbon nuclear translocator (ARNT), and this heterodimer activates a battery of genes that are under the control of the *Ah* gene locus [15,19,20]. CYP1A1 induction involves the interaction of the AHR–ligand–ARNT complex with multiple Ah-responsive elements (AHREs) within the *CYP1A1* gene promoter region to activate transcription [15,20–23]. The mechanisms involved in the hyperoxic induction of CYP1A1 are not fully understood, but an AHR-dependent mechanism has been proposed [16–20,24].

Whereas induction of CYP has been implicated in hyperoxic lung injury [25,26], several groups, including us have shown that CYP1A1 may play a protective role. Pretreatment of rats [27,28] or mice [29] with inducers of CYP1A enzymes attenuates hyperoxic lung injury. Additionally, we showed that pre-treating rats with a CYP1A-specific inhibitor, 1-aminobenzotriazole (ABT), followed by subsequent exposure to 95% O_2 severely potentiates hyperoxic lung injury [12]. These observations support the hypothesis that CYP1A1 and CYP1A2 may play protective roles against oxygen-mediated lung injury.

The mechanisms of induction of CYP1A1 by hyperoxia are not well understood. Therefore, in this study we tested the hypothesis that hyperoxia will induce transcriptional activation of human CYP1A1 or mouse CYP1A2 *in vivo* in transgenic mice carrying the human CYP1A1 or mouse CYP1A2 promoters, and that these mice display altered susceptibilities to hyperoxic lung injury.

2. Materials and methods

2.1. Chemicals

Sucrose, $NADP^+$, ethoxyresorufin, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma–Aldrich (St. Louis, MO). Polyvinylidene difluoride membranes and buffer components for electrophoresis and Western blotting were obtained from Bio-Rad Laboratories (Hercules, CA). The primary monoclonal antibody to CYP1A1 was a gift from Dr. P.E. Thomas (Rutgers University, Piscataway, NJ).

2.2. Animal studies and treatment protocol

Transgenic mice expressing the human 13.2 kb CYP1A1 promoter to drive luciferase expression (CYP1A1-luc) or mice expressing the 8.4 kb mouse CYP1A2 promoter (CYP1A2-luc) on CD-1 background were obtained from Xenogen Corporation (Alameda, CA) [27,28]. Twelve week-old male wild type (WT) (CD-1), CYP1A1-luc, or CYP1A2-luc mice were maintained in room air or exposed to hyperoxia (>95% O_2) for 24, 48, or 72 h, and luciferase expression was measured by bioluminescent imaging. After the imaging, the mice were sacrificed, and lung injury was assessed in three animals, and endogenous expression of CYP1A1 and 1A2 was determined in four different mice from each group.

2.3. Bioluminescent imaging

The substrate luciferin was administered into the peritoneal cavity at a dose of 150 mg/kg (20 mg/ml) approximately 5 min prior to imaging. Mice were anesthetized with isoflurane/oxygen and placed on the imaging station. Ventral and dorsal images were collected for 1 s using the IVIS imaging system 100 (Xenogen Corporation) [30], and luciferase expression was determined *in vivo* by bioluminescent imaging (Xenogen Corporation, Alameda, CA) [30–32]. Photons emitted from the liver region were quantified using Series Livingimage software [30–32].

2.4. Enzyme assays

Ethoxyresorufin *O*-deethylase (EROD) (CYP1A1) and methoxyresorufin *O*-deethylase (MROD) activities in the liver and lung microsomes were assayed essentially as described previously [9,17,18].

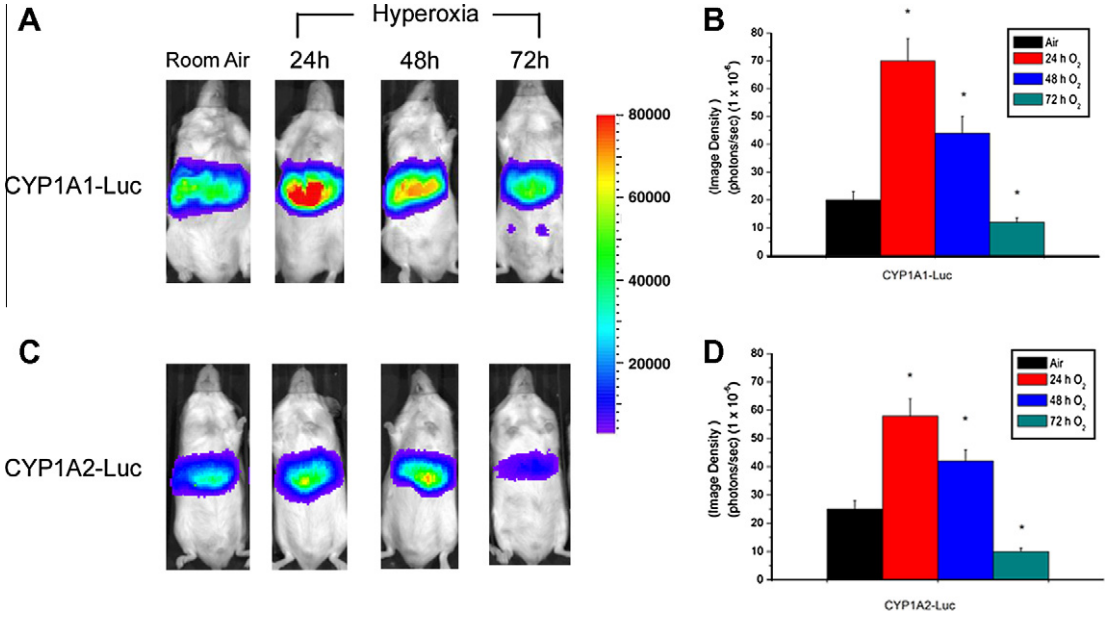

Fig. 1. Bioluminescent imaging of CYP1A1-luc (A, B) or CYP1A2-luc (C, D) mice following hyperoxia exposures. CYP1A1-luc (A) or 1A2-luc mice (C) were maintained in room air or exposed to hyperoxia (>95% O_2) for 24–72 h, and luciferase expression was analyzed by bioluminescent imaging in real time at the indicated time points. Quantitation of bioluminescent imaging data of CYP1A1-luc (B) or 1A2-luc (D) mice was conducted using IVIS imaging software. Values represent mean ± SE ($n = 5$). *Statistically significant differences between room air and hyperoxic mice at $P < 0.05$, as determined by two-way ANOVA.

2.5. Electrophoresis and Western blotting

Liver or lung microsomes (~20 μg) were subjected to SDS–polyacrylamide gel electrophoresis in 10% acrylamide gel followed by Western blotting to detect CYP1A1 and 1A2 proteins, as reported in the recent articles from our laboratory [9,17,18].

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

Total RNA (500 ng) from normoxic and hyperoxic mice was subjected to one step real time quantitative TaqMan RT-PCR. Gene-specific primers in the presence of TaqMan reverse transcription reagents and RT reaction mix (Applied Biosystems) were used to reverse transcribe RNA, and TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems) were used for PCR amplification, as reported earlier [18,31,32]. The relative mRNA levels for P4501A1 were normalized to their 18S content, and relative expression levels of CYP1A1 and 1A2 genes were calculated as reported earlier [18,31,32].

2.7. Lung injury

Lung injury was assessed by histology, as described previously [17,28].

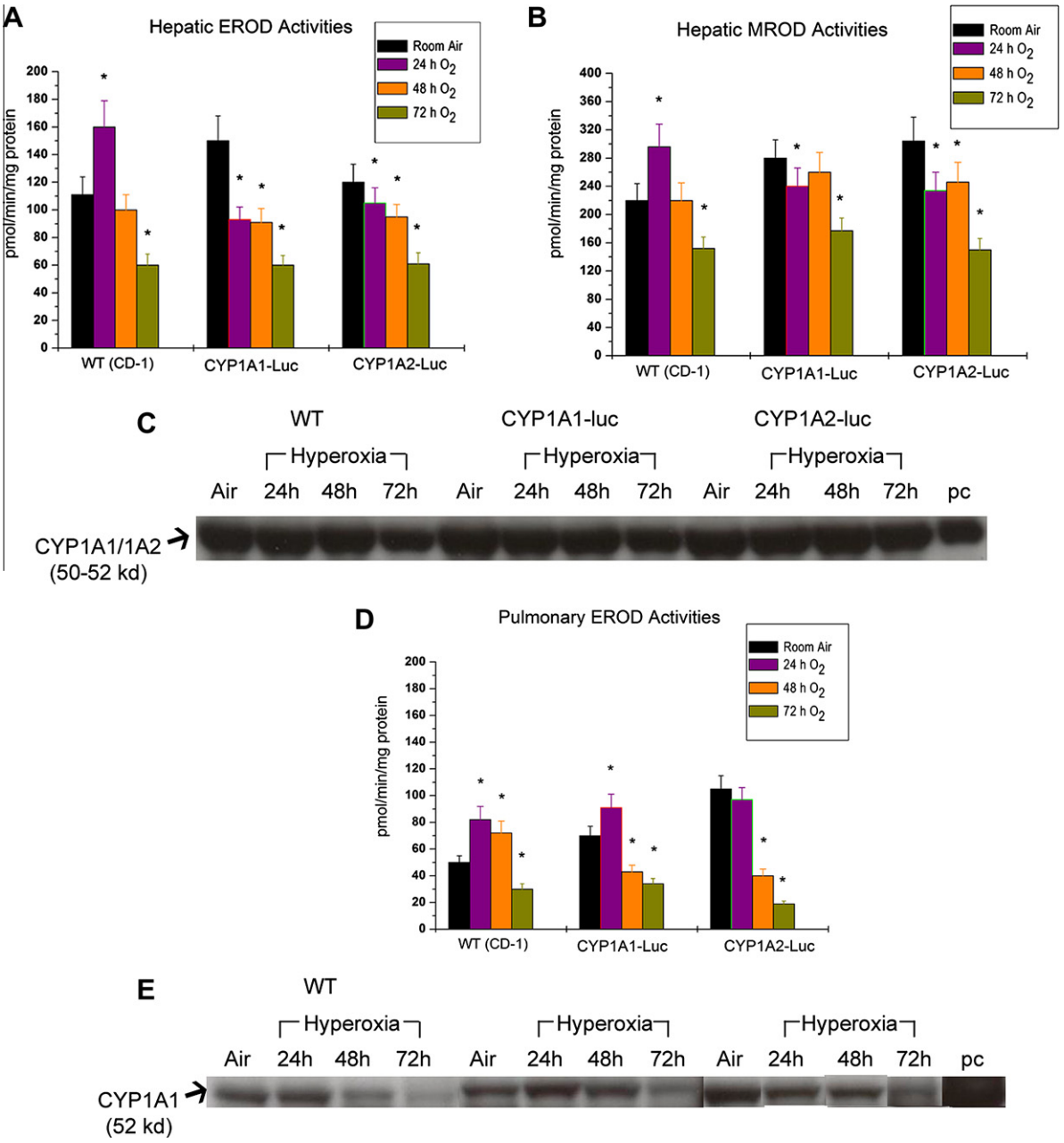

Fig. 2. Effect of hyperoxia on endogenous hepatic EROD (CYP1A1) (A), MROD (B), activities and apoprotein contents (C), and pulmonary EROD (D) and apoprotein expression (E). Adult male WT (CD-1), CYP1A1-luc or CYP1A2-luc mice were maintained in room air or exposed to hyperoxia at the indicated time points, and hepatic EROD (A), MROD (B) activities were determined in the liver microsomes. Data represent mean ± SE of fold induction versus room air controls from at least four individual animals. (C) Representative Western blot showing effect of hyperoxia on endogenous hepatic CYP1A1 and 1A2 (C) expression in mice. PC, positive control showing induction of CYP1A1/1A2 by 3-methylcholanthrene. (D, E) represent pulmonary EROD and apoprotein expression, respectively, in microsomes of WT, CYP1A1-luc, and CYP1A2-luc mice.

2.8. Statistical analyses

All statistical analyses were conducted using two-way ANOVA, followed by modified *t*-tests.

3. Results

3.1. Effect of hyperoxia on CYP1A1/1A2 promoter activation (luciferase reporter expression)

The major goal of this investigation was to determine the mechanisms of induction of CYP1A1 and 1A2 by hyperoxia *in vivo*. As shown in Fig. 1, hyperoxia exposure for 24–48 h resulted in marked induction of CYP1A1 (Fig. 1A and B)- and CYP1A2 (Fig. 1C and D)-promoter driven luciferase expression *in vivo* in the transgenic mice, compared to those maintained in room air. Luciferase expression declined after 72 h of hyperoxia (Fig. 1A–D). Quantitative imaging analyses showed that hyperoxia for 24 and 48 h induced CYP1A1-driven luciferase activities by 2.5- and 1.25-fold, respectively, over the corresponding room air controls (Fig. 1B). By 72 h, the luciferase expression was significantly lower than room air controls. In CYP1A2-luc mice, hyperoxia induced luciferase activities by 1.3- and 0.76-fold at the 24 and 48 h time points, respectively, over the corresponding room controls (Fig. 1D). Similar to the responses noted in CYP1A1-luc mice, hyperoxia for 72 h significantly attenuated CYP1A2-driven luciferase activities, compared to room air controls (Fig. 1D).

3.2. Effect of MC on endogenous CYP1A1/1A2 enzyme activities and apoprotein contents

In order to determine if CYP1A1-luc or CYP1A2-luc mice will display altered endogenous expression of CYP1A1 and/or 1A2, we determined the activities of EROD and MROD, reflecting catalytic functionalities of CYP1A1 and 1A2, respectively (Fig. 2). We also determined the CYP1A1 and 1A2 apoprotein contents. As shown in Fig. 2A, hyperoxia elicited a 45% induction of hepatic EROD activities in the WT (CD-1) mice at the 24 h time point. EROD activities declined by 48 h, and were significantly lower than room air controls after 72 h of hyperoxia (Fig. 2A). In CYP1A1-luc mice, room air controls displayed significantly higher expression of EROD than WT mice (Fig. 2A). Hyperoxia exposure resulted in suppression of EROD activities by 45%, 45%, and 60% after for 24, 48, or 72 h, respectively (Fig. 2A). CYP1A2-luc mice also showed similar trends, with hyperoxia diminishing the EROD activities at 24, 48, and 72 h, albeit to a lesser extent than that observed in the CYP1A1-luc mice (Fig. 2A).

Hepatic MROD, which reflects CYP1A2 activity, was also induced by hyperoxia by about 36% at 24 h. The activity declined by 48 h, and was significantly lesser than room air controls by 72 h (Fig. 2B). MROD activities were higher in the air-breathing CYP1A1-luc and CYP1A2-luc mice, compared to the WT controls (Fig. 2B). Hyperoxia exposure suppressed the MROD activities in the CYP1A1-luc, as well as CYP1A2-luc mice (Fig. 2B). Western blot analyses showed expression patterns of CYP1A1 and 1A2 (Fig. 2C) that correlated with enzyme activity data.

In pulmonary tissues, hyperoxia elicited a 0.6- and 0.4-fold induction of EROD (CYP1A1) activity at the 24 h and 48 time points, respectively, over room air controls in the WT mice (Fig. 2D), but the activities were suppressed (40%) after 72 h of hyperoxia (Fig. 2D). In CYP1A1-luc mice, hyperoxia for 24 h induced EROD activities by 28%, but after 48–72 h, the activities were significantly suppressed (43–46%) compared to room air controls (Fig. 2D). CYP1A2-luc mice showed the highest EROD activities in room air (Fig. 2D), but hyperoxia for 48 and 72 h diminished CYP1A1 activities by 68% and 88%, respectively, compared to room air controls (Fig. 2D). Western blot analyses showed that the CYP1A1 apoprotein contents correlated with enzyme activities (Fig. 2E).

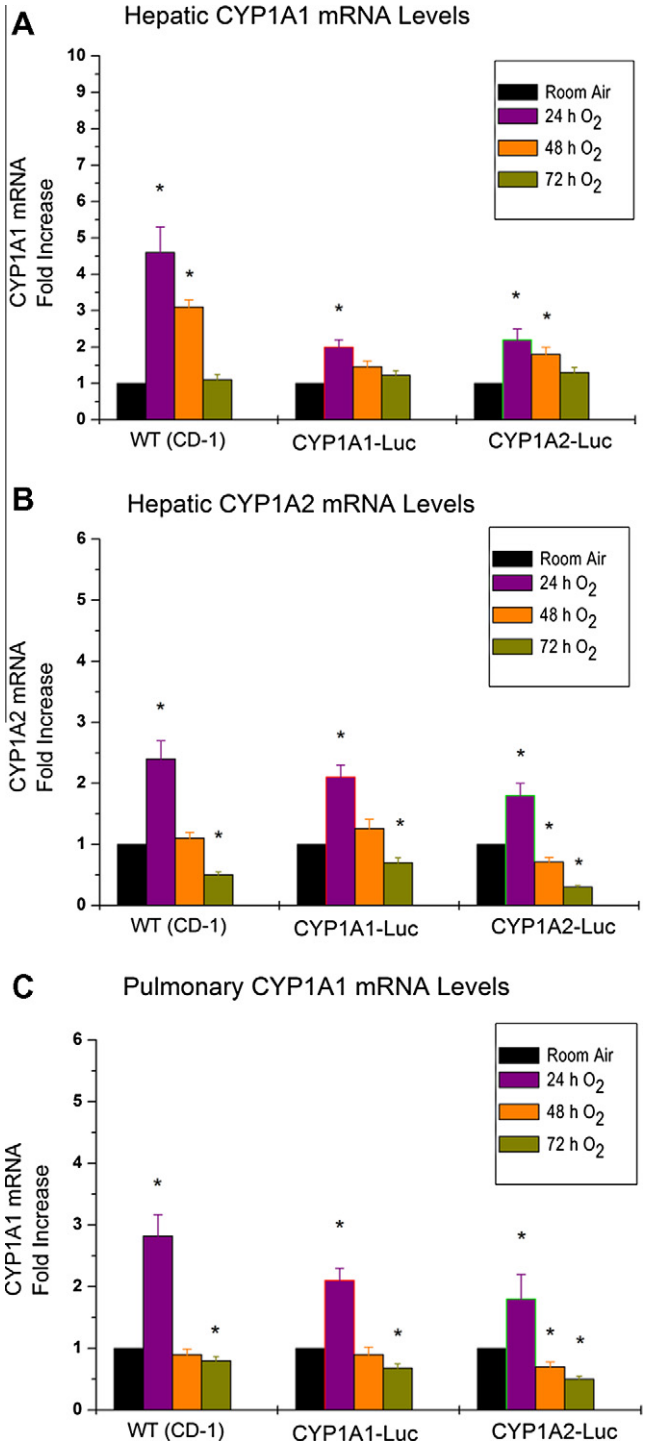

Fig. 3. Effect of hyperoxia on endogenous hepatic CYP1A1 (A), CYP1A2 (B), and pulmonary mRNA (C) in mice. Adult male WT (CD-1), CYP1A1-luc or CYP1A2-luc mice were maintained in room air or exposed to hyperoxia for 24–72 h, and hepatic CYP1A1 (A), CYP1A2 (B), and pulmonary CYP1A1 (C) mRNA levels were determined by real time RT-PCR at the indicated time points. Data represents mean ± SE of fold induction versus controls from at least four individual animals. *Statistically significant differences between room air and hyperoxic mice at $P < 0.05$, as determined by two-way ANOVA.

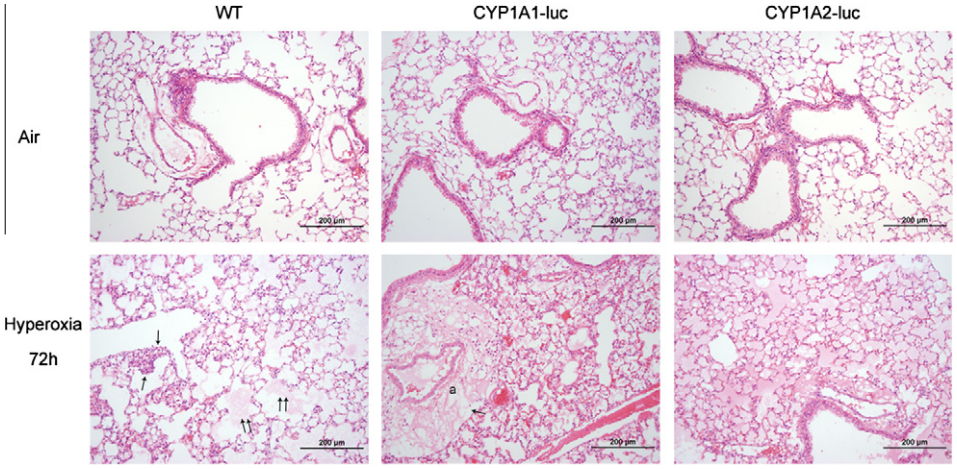

Fig. 4. Effect of hyperoxia on lung injury in WT, CYP1A1-Luc, and CYP1A2-mice. WT, CYP1A1-luc, and CYP1A2-luc mice were exposed to hyperoxia as described under Section 2, and lung tissues were processed for histological analyses. The figure shows representative light micrographs of lung sections of mice maintained in room air or exposed to hyperoxia for 72 h. Air-breathing mice (upper panels) showed normal lung architecture. Hyperoxia for 72 h showed increased lung injury in WT mice. Single arrow shows infiltration of inflammatory cells, and double arrow shows areas of intra-alveolar edema. CYP1A1-luc mice showed even more injury after 72 h of hyperoxia, with lungs showing perivascular and intra-alveolar edema and marked widening of the adventitia of the pulmonary artery. CYP1A2-luc mice showed the greatest susceptibility to lung injury, as evidenced by intra-alveolar edema and proteinaceous materials filling the alveoli.

3.3. Effect of hyperoxia on CYP1A1 and 1A2 mRNA expression

In WT mice, hyperoxia caused a 3.5- and 2-fold induction of hepatic CYP1A1 mRNA levels at the 1 and 8 days, respectively, versus controls (Fig. 3A). Induction declined by 72 h. In the CYP1A1-luc mice, the extent of induction of endogenous CYP1A1 mRNA after 24 h of hyperoxia was lesser than that observed in the WT mice, and by 48 h, the CYP1A1 levels declined to control (Fig. 3A). In the CYP1A2-luc mice, the degree of induction by hyperoxia was modest, being 1-, 0.6-fold, respectively, at the 24 and 48 h time points, respectively (Fig. 3A).

In contrast to CYP1A1, where hyperoxia for 24 h differentially induced this gene in WT and transgenic mice, hepatic CYP1A2 mRNA expression was augmented in the hyperoxic WT, CYP1A1-luc, and CYP1A2 mice to similar levels (Fig. 3B). In each of the genotypes, i.e. WT, CYP1A1-luc, and CYP1A2-luc mice, hyperoxia for 72 h caused a marked decline in CYP1A2 mRNA expression, compared to the corresponding air-breathing controls (Fig. 3B).

Similar to our observations in liver, hyperoxia caused a much greater induction in the levels of pulmonary CYP1A1 mRNA in the WT mice than the CYP1A1-luc or CYP1A2-luc mice (Fig. 3C). In WT mice, hyperoxia-exposed samples showed an induction of about 2-fold compared to controls at the 24 h time point. The induction declined by 48 h of hyperoxia, and was even more suppressed by 72 h (Fig. 3C). In CYP1A1-luc mice, hyperoxia caused a 1-fold increase at the 24 h time point over room air controls. Induction declined after 48 and 72 h time points of hyperoxia (Fig. 3C). In CYP1A2-luc mice, hyperoxia elicited induction of CYP1A1 mRNA expression at 24 h and significantly suppressed it by 48 h (Fig. 3C).

3.4. Effect of hyperoxia on lung injury

Hyperoxia for 24 or 48 h did not increase pulmonary edema in the WT mice as shown by lung weight/body weight ratios, or by histology (not shown). After 72 h of hyperoxia WT mice showed increased cellularity, infiltration of inflammatory cells, and intra-alveolar edema and (Fig. 4). CYP1A1-luc mice were much more susceptible to hyperoxic lung injury than similarly exposed WT mice (Fig. 4). CYP1A1-luc showed injury even at 48 h (not shown), and by 72 h these mice displayed perivascular inflammation, intra-alveolar edema, and widening of the adventitia of the pulmonary artery (Fig. 4). CYP1A2-luc mice showed the greatest lung injury after 72 h, with widespread intra-alveolar edema and proteinaceous material filling the alveoli (Fig. 4).

4. Discussion

In this study, we tested the hypothesis that hyperoxia induces human CYP1A1 and 1A2 enzymes by transcriptional activation of the corresponding promoters *in vivo*, and transgenic mice expressing the human CYP1A1 or 1A2 promoters would be more susceptible to hyperoxic lung injury than WT mice.

Our results showing marked induction of luciferase expression in transgenic mice expressing CYP1A1-luc or 1A2-luc (Fig. 1A–D), supporting the hypothesis that hyperoxia elicited CYP1A induction by transcriptional activation of the corresponding promoters. The observation that hyperoxia elicited greater degree induction of luciferase expression in the CYP1A1-luc mice compared to that of CYP1A2-luc mice was similar to our previous findings on the induction of CYP1A1 and 1A2 by 3-methylcholanthrene [30,31]. In fact, we show for the first time that hyperoxia induces CYP1A genes by transcriptional activation of the CYP1A promoters *in vivo*. Thus, these transgenic models can be very useful in determining the mechanisms of induction of CYP1A *in vivo*.

The finding that hyperoxia caused marked increases of hepatic EROD (Fig. 2A) and MROD activities (Fig. 2B) in the WT mice indicated induction of CYP1A1 and 1A2 expression, as EROD and MROD activities are known to reflect CYP1A1 and 1A2 activities, respectively [9,17,18]. These results were consistent with our previous findings showing induction of CYP1A1 enzymes in rats and mice by hyperoxia *in vivo* [9,17,18].

The fact that hyperoxia induced the endogenous CYP1A1 and 1A2 activities in the transgenic mice suggests that introduction of the promoters into the mice did not affect the phenomenon of induction, and that the molecular properties governing gene expression of the CYP1A-luc mice and the endogenous CYP1A genes are similar [31–33].

That hyperoxia also caused induction of CYP1A1 and 1A2 apoproteins in liver (Fig. 2A and B), and CYP1A1 alone in the lungs of WT and transgenic mice was in agreement with our enzyme activity data. The results in Fig. 3A showing a robust induction of hepatic CYP1A1 mRNA in the WT mice was consistent with the

hypothesis that hyperoxia induced CYP1A1 activity and protein expression by inducing its message [18,24]. The fact that hyperoxia caused much lesser induction of CYP1A1 mRNA in the livers of CYP1A1-luc and CYP1A2-luc mice was probably due to decreased accessibility of the ligand–AHR–ARNT complex and other co-activators to the endogenous CYP1A1 enhancer elements, presumably through alteration in chromatin remodeling as a result of incorporation of the CYP1A1 promoter transgene.

The fact that hyperoxia for 24 h elicited induction of hepatic CYP1A2 expression in the CYP1A1-luc similar to that in WT mice (Fig. 3B) supported the hypothesis that introduction of human CYP1A1 promoter into the genome of mice did not alter the susceptibility of CYP1A2 gene to hyperoxic induction. On the other hand the diminution of CYP1A2 mRNA induction in the CYP1A2-luc mice may have been due to decreased accessibility of the ligand–AHR–ARNT complex to CYP1A2 enhancer(s) that are responsible for induction of the CYP1A2 mRNA [31]. These results also suggest that mechanisms governing transcriptional activation of exogenous CYP1A promoters by hyperoxia are similar to those of the endogenous counterparts.

The increased susceptibility of CYP1A1-luc and 1A2-luc to hyperoxic lung injury was probably due to decreased endogenous expression of CYP1A enzymes in these mice by prolonged hyperoxia. Our previous results showed that CYP1A enzymes play beneficial roles against hyperoxic lung injury by detoxifying ROS such as F_2-isoprostanes [34]. F_2-isoprostanes have been shown to be elevated in rats exposed to hyperoxia [35], and these products are formed by reactive oxygenase species mediated peroxidation of lipid hydroperoxides, resulting in protein and DNA oxidation, eventually leading to lung injury and cell death [10]. In fact, levels of F_2-isoprostanes are elevated in premature infants suffering from BPD [36], suggesting that these compounds may have an etiologic role in lung injury.

Thus, further studies on the molecular regulation of CYP1A genes by hyperoxia could lead to novel strategies for the prevention and/or treatment of BPD in premature infants and ARDS in adults.

Acknowledgments

We thank Dr. Paul Thomas (Rutgers University) for providing us monoclonal antibodies against CYP1A1/1A2.

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