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ATTENUATION OF OXYGEN-INDUCED ABNORMAL LUNG MATURATION IN RATS BY RETINOIC ACID: POSSIBLE ROLE OF CYTOCHROME P4501A ENZYMES

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Abbreviations: BPD, bronchopulmonary dysplasia; ROS, reactive oxygen species; CYP, cytochrome P450; AHR, Ah receptor; EROD, ethoxyresourufin O-deethylase; MROD, methoxyresorufin O-demethylase; ANOVA, analyses of variance; AHREs, Ah response elements; ARNT, Ah receptor nuclear translocator; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; H & E, hematoxylin & eosin; RA, retinoic acid; RAR; retinoic acid receptor; RXR, retinoic acid X receptor; VEGF, vascular endothelial growth factor.

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ABSTRACT

Supplemental oxygen is frequently used in the treatment of infants having pulmonary insufficiency, but prolonged hyperoxia may contribute to the development of bronchopulmonary dysplasia (BPD) in these infants. Cytochrome P450 (CYP) 1A enzymes have been implicated in hyperoxic lung injury. Retinoic acid (RA) plays a key role in lung development. Here, we tested the hypotheses that newborn rats exposed to a combination of RA and hyperoxia would be less susceptible to lung injury than those exposed to hyperoxia only, and that modulation of CYP1A enzymes by RA contribute to the beneficial effects of RA against hyperoxic lung injury. Newborn rats exposed to hyperoxia for 7 days showed higher lung weight/body weight (LW/BW) ratios compared to those exposed to RA + hyperoxia. Hyperoxia for 7 days also caused a significant increase in hepatic and pulmonary CYP1A1/1A2 expression compared to air-breathing controls. RA + hyperoxia treatment lowered the expression of these genes. Seven to 30 days after withdrawal of hyperoxia, the animals showed marked induction of hepatic and pulmonary CYP1A1/1A2 expression, but animals that had been given RA + hyperoxia displayed lower expression of these enzymes. On postnatal days (PND) 22 or 38, the hyperoxic animals displayed retarded lung alveolarization; however, the RA + hyperoxia-exposed animals showed improved alveolarization. The improved alveolarization in animals given RA + hyperoxia, in conjunction with the attenuation of CYP1A1 and 1A2 expression in these animals suggests that this phenomenon may play a role in the beneficial effects of RA.

Introduction

Supplemental oxygen therapy is frequently used in preterm and term infants and in adults with acute respiratory distress syndrome (Northway and Rosan, 1968). Considerable evidence links oxygen to the development of bronchopulmonary dysplasia (BPD) in premature infants (Tsai et al., 1972). Exposure of experimental animals to hyperoxia causes lung damage (Frank, 1991; Couroucli et al., 2002; Jiang et al., 2004). Hyperoxia exposure in the newborn rodents leads to arrested alveolarization and abnormal lung maturation in adulthood (Frank, 1991; Lin et al., 2005; Bourbon et al., 2005). The molecular mechanisms responsible for oxygen toxicity are not completely understood, but reactive oxygen species (ROS) have been implicated (Frank, 1991).

Cytochrome P450 (CYP) enzymes are a superfamily of hemoproteins that metabolize a large number of endogenous and exogenous compounds through mechanisms that include oxidation, reduction, and peroxidation (Guengerich, 1990). Among these, the CYP1A enzymes are of particular interest to oxygen toxicity, as indicated by differential susceptibilities of aryl hydrocarbon (*Ah)-responsive* mice and *Ah-nonresponsive* mice to oxygen-induced lung injury (Gonder et al., 1985). Exposure of adult rats to hyperoxia for 48 h leads to induction of CYP1A enzymes in liver and lung (Okamaoto et al., 1993; Moorthy et al., 1997; Couroucli et al., 2002), Interestingly, the induction of CYP1A enzymes in liver and lung declines after continuation of hyperoxia for 60 h, the time period that coincides with overt respiratory distress in these animals, suggesting that decline of CYP1A enzyme induction contributes to hyperoxic lung injury (Moorthy et al., 1997; 2000; Couroucli et al., 2002). Mansour et al. (1988a; 1988b) observed protection against hyperoxia-induced lung injury by pretreatment of adult rats or mice with the CYP1A1 inducer 3-methylcholanthrene (MC). We recently showed that the CYP1A inducer β-

naphthoflavone protects adult rats against hyperoxic lung damage (Sinha et al., 2005). Adult mice deficient in the gene for the Ah receptor (AHR) (Jiang et al., 2004) or the liver-specific CYP1A2 (Moorthy et al., 2005) are more susceptible to hyperoxic lung injury than wild type mice, supporting the hypothesis that the CYP1A enzymes play a beneficial role against lung injury in adult animals. Since CYP1A2 is specifically expressed in the liver, it is possible that hepatic CYP1A enzymes also play important role(s) in the effects of hyperoxia (Moorthy et al., 2005). In contrast, oxygen-induced lung damage in neonatal rats is potentiated by pretreatment with the CYP1A inducer MC (Theibault et al., 1991). The paradoxical effects of CYP1A inducers on hyperoxic lung injury in adult and newborn rats strongly suggest that the developmental status of the animal significantly influences the susceptibility of the organism to modulation by CYP1A inducers.

Retinoic acid (RA) and its synthetic analogs are potent regulators of a diverse group of biological processes, including growth, differentiation, cell proliferation, and morphogenesis (Gudas et al., 1994). The biological effects of RA and its synthetic analogs are mediated by RA receptors (RARs) and RXRs (Chambon, 1996; Kimura et al., 2002). The RARs and RXRs are RA-inducible transcriptional regulatory proteins that regulate gene expression via specific cis-acting DNA sequences [retinoic acid response elements (RAREs)] located in the promoters of target genes. RA may modulate CYP1A1 gene expression through retinoid receptors or through the AHR (Suprano et al., 2001).

Recent work has suggested that RA plays a key role in induction of formation of septa during lung alveolarization (Massaro and Massaro, 2002). Furthermore, it has been recently reported that early lung bud formation and subsequent branching and morphogenesis are characterized by distinct stages of RA signaling. If alveolarization is compromised at this stage

by exposure to hyperoxia or other insults, the changes persist into adulthood. These observations are of clinical significance because a similar type of altered lung development (retarded alveolarization) is seen in infants who develop BPD (Margraf et al., 1991). Recently, Veness-Meehan et al. (2002) and Ozer et al. (2005) have reported that RA treatment during hyperoxia has beneficial effects on lung alveolarization, but the mechanisms are not understood. Randomized double-blinded clinical trails have suggested that vitamin A supplementation in extremely low birth weight infants might decrease the risk for chronic lung disease (Tyson et al., 1999).

Since CYP1A enzymes in the newborn animals appear to play important roles in lung injury, and because RA may modulate CYP1A expression, in this investigation, we tested the hypothesis that pretreatment of newborn rats with RA, prior to exposure to hyperoxia would protect animals from oxygen-induced abnormal lung maturation during adulthood and that modulation of pulmonary as well as hepatic CYP1A enzymes contribute to the beneficial effects of RA.

Materials and Methods

Animals. Timed pregnant newborn Fisher 344 rats were purchased from Charles River. Newborn rats were delivered from these mothers. Newborn rats were treated i.p. with RA (0.5 mg/kg) or vehicle [(corn oil (CO)], once daily for 5 days and were either maintained in room air or placed in oxygen chambers (> 95% O_2) immediately after the first RA treatment. Exposure to hyperoxia was continued for 7 days, and the animals were either sacrificed immediately (PND 8) or were returned to room air and were sacrificed on PND 15, 22, or 38. It was ensured that minimum air exposure (less than 5 min) occurred when hyperoxic animals were treated with RA from day 2 to day 5. Lung injury was analyzed measuring ratios of lung weight to body weight (LW/BW) and by histology. All animal experiments were carried out in accordance to the Guide for the Care and Use of Laboratory Animals as adopted and promulugated by the U.S. National Institutes of Health. The experiments reported herein were reviewed and approved by the Baylor Institutional Animal Care and Use Committee.

Hyperoxia Exposure. The newborn animals were either maintained in room air or exposed to > 95 % O_2 for 7 days using pure O_2 at 5 L/min, as we have described previously (Couroucli et al., 2002). The dams were rotated between hyperoxic and room air chambers once every 24 h to prevent toxicity to the mothers.

Perfusion and tissue harvesting. At the termination of their respective exposures, 8 rats from each group were anesthetized with sodium pentobarbital (200 mg/kg), i.p. and euthanized by exsanguination while under deep pentobarbital anesthesia. The lungs were perfused with phosphate buffered saline, and microsomes were prepared for subsequent analyses of CYP1A1-dependent activities and immunoreactive protein contents in individual animals. The livers were

also obtained for CYP1A1/1A2 analyses. For histological studies, the left lungs were inflated through the intratracheal catheter and were fixed at constant pressure (20 cm H_2O) with zinc formalin after which the lungs were embedded in paraffin for subsequent histological analyses for assessing lung injury (Couroucli et al, 2002). The right lungs were used for subsequent RNA isolation and analyses.

Chemicals. Calcium chloride, Tris, sucrose, NADPH, bovine serum albumin, ethoxyresorufin, glutathione reductase, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase 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 (Thomas et al., 1984), was a generous gift from Dr. P.E. Thomas. Goat anti-mouse IgG conjugated with horseradish peroxidase was from Bio-Rad laboratories (Richmond, CA).

Preparation of Microsomes and Enzyme Assays. Lungs and livers were perfused with ice-cold phosphate-buffered saline, pH 7.4. Lung microsomes were prepared by differential centrifugation, as reported previously (Couroucli et al., 2002) from individual animals. Liver microsomes were isolated by the calcium chloride precipitation method (Moorthy et al., 1997). Protein concentrations were estimated by the Bradford dye-binding method (Bradford, 1976). Ethoxyresorufin O-deethylase (EROD) (CYP1A1) activities in lung and liver microsomes and methoxyresorufin O-demethylase (MROD) (CYP1A2) activities in liver microsomes were assayed as we have described previously (Moorthy et al., 1997).

Western blotting. Liver microsomes (20 μg of protein) prepared from individual animals 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 (Moorthy et al., 1997; 2000; Couroucli et al., 2002).

Reverse transcriptase-polymerase chain reaction (RT-PCR) Assays. Total RNA (20 μg) from livers of air-breathing and hyperoxic animals was reverse-transcribed (Wang and Strobel, 1998; Couroucli et al., 2002), and the resulting cDNA was used as template for PCR analysis. Primers specific for CYP1A1 (5' GGCCAGACCTCTCTACAGTTC-3') and 5' GCCAAGCATATGGCACAG-3'); and cyclophilin (CYC) (5' CGAGCTTTTTGCAGCCAAAG 3' and 5' AGCCACTCAGTCTTGGCAGT 3'), as internal control, were used in PCR reactions to amplify the corresponding cDNAs made in the reverse transcriptase step (Wang and Strobel, 1998; Couroucli e al., 2002).

Southern Blot Analysis of PCR Products. The PCR products, generated by PCR amplification of cDNA for 35 cycles, were separated on 1% agarose gel, transferred to nylon membranes by capillary blotting, and probed with random prime labeled cDNA probes for CYP1A1 or cyclophilin (CYC), which were prepared by PCR amplification, followed by purification and extraction of the PCR-products from agarose gels (Wang and Strobel, 1998). The membranes were exposed to a phosphor-imager, and pixel densities of the PCR products were measured (Couroucli et al., 2002).

Lung weight/body weight ratios. 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.

Lung histology. Routine histology was performed on lung tissues from individual animals as described previously (Couroucli et al., 2002; Jiang et al., 2004; Sinha et al., 2005).

Statistical Analyses. Data are expressed as means ± SE. Two-way analyses of variance (ANOVA), followed by modified t-tests, were used to assess significant differences arising from

exposure to hyperoxia and RA for different time points. *P* values < 0.05 were considered significant.

Results

In this investigation, we studied the effects of hyperoxia and RA on lung injury and CYP1A expression in the newborn rat. Newborn rats exposed to hyperoxia for 7 days showed higher LW/BW ratios at 1 day compared to those exposed to RA + hyperoxia, CO + air, or RA + air (Figure 1). While the LW/BW ratios were elevated (30%) in the CO + hyperoxia group even 7 days after return of the animals to room air, fifteen to thirty days after return of animals to room air (PND 22 or 383), the CO + hyperoxia animals did not show any alterations in the LW/BW ratios compared to any of the other groups (Figure 1).

When the lungs were examined histologically, the air-breathing animals treated with the vehicle CO or RA on PND 8 or 15 showed normal lung structure, and there was no evidence of tissue injury (Figure 2A and B, panels a and c) . After 7 days of hyperoxia (Figure 2A, panel b), the lungs showed pulmonary edema and perivascular inflammation. Animals given a combination of hyperoxia and RA showed lesser lung injury (Figure 2A, panel d) than those exposed to hyperoxia alone. Seven days after return to room air, animals given CO + hyperoxia (Figure 2B, panel b) still showed acute lung damage, although it was not as pronounced as that seen when animals were sacrificed 7 days after hyperoxia. Even at this time point, the RA + hyperoxia group showed lesser lung damage (Figure 2B, panel d).

Since lung development in the rat continues through adulthood, we examined the lungs of rats 15 (PND 22) or 30 (PND 38) days after return of the hyperoxic animals to room air. On PND 22, the air-breathing animals treated that been treated with the vehicle CO or RA during the neonatal period showed more alveolar septation compared to those examined at PND 8 or 15 (Figure 3A, panels a and c). By PND 38, the lung architecture of these animals appeared normal (Figure 3B, panels a and c). On the other hand, the oxygen-exposed animals on PND 22 (Figure

3A, panel b) or PND 38 (Figure 3B, panel b), showed marked enlargement of alveolar spaces, widened distal airspace, and lesser septation. In addition, free alveolar macrophages were present in the lungs of these animals. Animals exposed to a combination of RA and hyperoxia showed improved septation of the alveoli compared to those exposed to oxygen only on PND 22 (Figure 3A, panel d) as well as PND 38 (Figure 3B, panel d). RA improved distal lung structure, as reflected by smaller and more numerous alveoli (Figure 3A and B, panel d). However, some perivascular inflammation was observed in the RA+ hyperoxia samples (Figure 3A and B, panel d).

In order to study the possible relationship between lung damage and CYP1A expression, we determined the effects of RA and hyperoxia on pulmonary CYP1A1 and hepatic CYP1A1 and 1A2 expression. Hyperoxia alone for 7 days caused a 3-fold increase in pulmonary EROD (CYP1A1) activities compared to air-breathing animals (Figure 4). RA + hyperoxia samples also showed significant induction of CYP1A1 activities on PND 8 (Figure 4). However, RA administration to air-breathing animals did not significantly alter the expression of CYP1A1 (Figure 4). Seven to 30 days after return of the animals to room air, the CO + hyperoxia, but not RA + hyperoxia, animals displayed a sustained induction (1.5-3-fold) of CYP1A1 activities over room air animals (Figure 4). Interestingly, the RA + hyperoxia samples displayed a 50% decrease in CYP1A1 expression compared to air-breathing animals treated with the vehicle CO or RA on PND 38 (Figure 4). Western blot analyses of pulmonary CYP1A1 revealed that the modulation of protein expression by hyperoxia and/or RA followed trends that were similar to those observed with the corresponding enzyme activities data (Figure 5).

In order to determine if modulation of CYP1A1 enzyme expression by hyperoxia and/or RA was accompanied by similar alterations in the expression of the corresponding mRNA, we

performed RT-PCR, followed by semi-quantiative analyses of the mRNA expression by PCR-Southern analyses. Hyperoxia for 7 days induced CYP1A1 mRNA by 1.3-fold compared to air-breathing animals (Figure 6, Table 1). This induction was sustained through PND 38 (Figure 6, Table 1). RA + hyperoxia attenuated CYP1A1 expression compared to CO + hyperoxia samples at each of the time points (Figure 6, Table 1).

Because we have previously observed significant modulation of hepatic CYP1A enzymes by hyperoxia (Moorthy et al., 1997; 2000; Couroucli et al., 2002; Jiang et al., 2004; Sinha et al., 2005), we conducted experiments to determine the effect of hyperoxia and RA on hepatic EROD (CYP1A1) and MROD (CYP1A2) activities. Hyperoxia caused a 2-fold induction of hepatic EROD after 7 days, compared to room air animals (Figure 7). RA + hyperoxia samples did not elicit any significant changes in EROD activities compared to air-breathing animals, but were lower than the CO + hyperoxia group (Figure 7). The hyperoxia-mediated induction of CYP1A1 persisted through PND 38 (Figure 7). RA by itself did not alter CYP1A1 expression at PND 38, but RA + hyperoxia attenuated CYP1A1 expression. Similar results were observed regarding the effects of hyperoxia and RA on MROD activities (Figure 8). The western blot analyses revealed that the protein expression was in agreement with EROD and MROD activity data (Figure 9).

In order to determine if modulation of hepatic CYP1A1/1A2 enzyme expression by hyperoxia and/or RA was accompanied by similar alterations in the expression of the corresponding mRNAs, we performed RT-PCR, followed by semi-quantiative analyses of the mRNA expression by PCR-Southern analyses. Hyperoxia for 7 days induced CYP1A1 (Figure 10) and 1A2 mRNA (Figure 11) by 1.3-1.7-fold compared to air-breathing animals (Table 2). This induction was sustained through PND 38 (Figures 10,11, Table 2). RA, by itself, did not significantly alter the expression of hepatic CYP1A2 or 1A2 at either time point. Animals that

were pretreated with RA, prior to hyperoxia exposure also did not elicit any changes in the expression of CYP1A1/1A2 mRNAs compared to air-breathing animals that were treated with vehicle. The levels of these mRNAs in the RA+ hyperoxia were lower than the CO + hyperoxia group, however (Figures 10,11 Table 2).

Discussion

In this investigation we tested the hypothesis that exposure animals to a combination of RA and hyperoxia would protect rats abnormal lung maturation by hyperoxia alone, and that CYP1A1 would play a role in this phenomenon. The increase the LW/BW ratios in hyperoxic animals at 7 days (Figure 1) and the histological evidence of lung damage (Figure 2) was in agreement with previous studies showing acute lung injury in newborn rats exposed to prolonged hyperoxia (Bucher and Roberts, 1981; Couroucli et al., 2006). The protection of lung injury by RA (Figures 1-3) pretreatment was consistent with the hypothesis that RA protects newborn animals from acute lung injury induced by oxygen.

The retarded alveolarization of animals 15-30 days after return of hyperoxic animals to room air (Figure 3) was in agreement with previous studies showing abnormal lung maturation in animals that had been exposed to hyperoxia during the newborn period (Frank, 1991; Bourbon et al., 2005; Lin et al., 2005). That RA pretreatment significantly improved lung alveolarization strongly suggests that RA protects against abnormal lung maturation by hyperoxia.

The marked increases (~3-fold) in lung EROD activities (Figure 4) caused by exposure to hyperoxia for 7 days indicate induction of CYP1A1, as EROD activities are relatively specific for CYP1A1 (Couroucli et al., 2002; Moorthy, 2000). Similar results were obtained in adult male Sprague-Dawley rats, wherein induction of pulmonary CYP1A1 activities was observed after 48 h of hyperoxia (Couroucli et al., 2002). Our observation that hyperoxic animals displayed a significant induction of pulmonary EROD activities even 30 days after return of the animals to room air suggests that neonatal hyperoxia causes long-term alterations in CYP1A1 gene expression. However, the RA + hyperoxia samples did not show CYP induction at either time point, suggesting that suppression of hyperoxia-mediated CYP1A1 induction by RA may

have played a role in the protection against acute lung injury and abnormal lung injury by hyperoxia.

The mechanisms underlying the prolonged CYP1A1 induction by hyperoxia are not clearly understood. The levels of CYP enzymes are very low at birth, and increase rapidly in a time-dependent manner, with the perinatal period showing a marked increase in CYP levels (Omeicinski et al., 1990). Exposure of animals to drugs and other foreign compounds (xenobiotics) has been shown to alter neonatal androgen levels during the critical neonatal period (Omeicinski et al., 1990) and cause permanent alterations in the expression of several CYP isozymes (imprinting) in the grown animals (Fujita et al., 1995). Because exposure of newborn animals to hyperoxia causes lung developmental abnormalities, we hypothesize that long-term alterations in the expression of specific CYP isoforms, caused by neonatal exposure of rats to hyperoxia, contributes mechanistically to the persistent developmental abnormalities that are observed in adult life.

We reported earlier that treatment of adult rats with the PAH 3-methylcholanthrene elicits persistent induction of hepatic and extrahepatic CYP1A enzymes for several weeks after PAH withdrawal (Moorthy, 2000). This persistent induction appears to occur by mechanisms independent of the persistence of the parent compound. Since newborn animals exposed to hyperoxia also display long-term induction, it is conceivable that these two phenomena may involve similar mechanisms.

The mechanisms of induction of CYP1A1 by PAHs have been extensively studied (Okey et al., 1994), and the AHR plays an important role in the induction process. We recently provided evidence that hyperoxia also induces CYP1A1 by AHR-mediated mechanisms (Couroucli et al., 2002: Jiang et al., 2004). Recent studies have suggested that AHR has

important physiological functions beyond mediating the response to environmental contaminants, such as liver development and immune function (Gambone et al., 2002; Rushing and Denison, 2002; Jiang et al., 2004). In fact, a number of naturally occurring compounds have been reported to be relatively weak AHR ligands (e.g., tryptophan metabolites, bilirubin, benzocoumarins, and substituted flavonoids (Gambone et al., 2002). Although several synthetic retinoids (e.g., AGN 193109, AGN 190730) can elevate CYP1A1 mRNA levels in mouse embryos and in Hepa-1c1c7 cells through the AHR/ARNT pathway (Gambone et al., 2002), RA does not induce CYP1A1. In fact, RA causes repression of AHR-induced CYP1A1 gene expression by mechanisms involving the SMRT corepressor (Fallone et al., 2004). Recent studies have also suggested cross-talk between the RAR/RXR with the AHR (Rushing and Denison, 2002), a phenomenon that could contribute to the attenuation of CYP1A expression by RA + hyperoxia in our experiments.

The lesser acute lung injury and improved alveolarization in animals given RA + hyperoxia versus those given oxygen only supports the hypothesis that RA ameliorates hyperoxic lung injury. Because CYP1A1 has been implicated in the generation of reactive oxygen species, the attenuation of pulmonary CYP1A1 expression by RA + hyperoxia suggests that RA may have ameliorated lung injury, in part, by downregulating CYP1A1 expression. The recent findings of Yang et al. (2005) showing suppression of TCDD-induced CYP1A1 expression through the repression of the AHR lends credence to the hypothesis that RA may have attenuated hyperoxia-induced CYP1A1 expression by mechanisms involving downregulation of the AHR. On the basis of our experiments and studies of other investigators, we have proposed a mechanism (Figure 12) which could explain the beneficial effects of RA. We postulate that hyperoxia-mediated induction of CYP1A enzymes in the newborn rat could lead to increased

ROS production, resulting in lung injury. RA, by attenuating CYP1A expression, and thereby decreasing ROS formation, prevents lung injury. The other possibility is that high CYP1A1 levels in the hyperoxic animals may have led to increased metabolism and elimination of RA (Choudary et al., 2004: Fletcher et al., 2005), leading to developmental abnormalities. Alternatively, the increased expression of CYP4F4, which plays a role in the catabolism of pro-inflammatory eicosanoids, by RA + hyperoxia may have contributed to the attenuation of lung injury by this agent.

The increases in pulmonary (Figure 4) and hepatic EROD activities (Figure 7) were paralleled by augmentation of CYP1A1/1A2 protein contents (Figures 5, 9) and mRNA levels (Figures 6,10,11) suggested that hyperoxia induced CYP1A enzymes by transcriptional or post-transcriptional mechanisms. The observation that hyperoxia induced hepatic MROD activities in the hyperoxic animals (Figure 8), a phenomenon that was paralleled by induction of CYP1A1/1A2 apoprotein (Figure 9) contents and the corresponding mRNA (Figures 10,11) suggested that induction of CYP1A2 by hyperoxia was also mediated by transcriptional or post-transcriptional mechanisms.

Recent studies have suggested that impaired angiogenesis due to inhibition of vascular endothelial growth factor (VEGF) signaling decreases alveolar growth in the developing lung, suggesting that impaired VEGF signaling may contribute to decreased lung growth in BPD (Kunig et al., 2005; Lin et al., 2005). In fact, Clerch et al. (2004) have shown that dexamethasone-induced inhibition of alveolar septation is associated with a block in angiogenesis due to downregulation of VEGF receptor-2, and that the downregulation of VEGR receptor-2 is prevented by treatment with RA. It is not known if there is a mechanistic link between CYP1A1, VEGF signaling, and lung development, but future studies along these lines

could contribute to the development of new interventions in the treatment of BPD in infants. Regardless of the mechanism by which RA appears to protect animals against oxygen-induced tissue damage, the present study provides conclusive evidence that RA does play a beneficial role in hyperoxic lung injury, and future work to identify the specific mechanisms of protection could lead to the development of rational strategies for the prevention/treatment of lung diseases in infants and adults undergoing supplemental oxygen therapy.

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Figure Legends

Figure 1. Effects of hyperoxia and RA + hyperoxia on LW/BW ratios in newborn rats. Male newborn Fisher rats were maintained in room air or exposed to hyperoxia for 7 days and animals were either sacrificed immediately (PND 8) or on PND 15, 22 or 38. Some animals were treated i.p. with RA (0.5 mg/kg) or vehicle [(corn oil (CO)], once daily for 5 days and were either maintained in room air or exposed to hyperoxia. LW/BW ratios were determined in these animals. Values represent means ± SE (n =4). Two-way ANOVA, followed by modified t-tests were used to assess statistically significance between individual groups. ^{a,b,c,d} denote significant differences from CO + air (a), CO + hyperoxia (b), RA + air (c), and RA + hyperoxia (d) at $P < 0.05$.

Figure 2. Comparative morphology by light photomicroscopy of representative lung sections from rats exposed to air or oxygen. Newborn rats were exposed to hyperoxia or hyperoxia + RA, as described under Materials and Methods, and animals were sacrificed on PND 8 (A) or 15 (B). a, Air-breathing controls (20x magnification); b, Oxygen-exposed animals; c, Air breathers treated with RA; and d, animals treated with RA + hyperoxia. Bar = 100 μm.

Figure 3. Comparative morphology by light photomicroscopy of representative lung sections from rats exposed to air or oxygen. Newborn rats were exposed to hyperoxia or hyperoxia + RA, as described under Materials and Methods, and animals were sacrificed on PND 22 (A) or 38 (B). a, Air-breathing controls (20x magnification); b, Oxygen-exposed animals; c, Air breathers treated with RA; and d, animals treated with RA + hyperoxia. Bar = 100 μm.

Figure 4. Effect of hyperoxia and RA on lung EROD (CYP1A1) activities. Male newborn Fisher rats were maintained in room air or exposed to hyperoxia for 7 days and animals were sacrificed on PND 8, 15, 22, or 38. Some animals were treated i.p. with RA (0.5 mg/kg) or vehicle [(corn oil (CO)], once daily for 5 days and were either maintained in room air or exposed to hyperoxia. EROD (CYP1A1) activities were determined in the lung microsomes. Values represent means ± SE (n =4). Two-way ANOVA, followed by modified t-tests were used to assess statistically significance between individual groups. ^{a,b,c,d} denote significant differences from CO + air (a), CO + hyperoxia (b), RA + air (c), and RA + hyperoxia (d) at $P < 0.05$.

Figure 5. Representative western blot showing the effect of hyperoxia and RA on lung CYP1A1 apoprotein. Rats were treated with hyperoxia and/or RA as described under Materials and Methods, and CYP1A1 apoprotein expression was determined in lung microsomes (20 μg) of these samples by Western blotting at the indicated time points.

Figure 6. RT-PCR analysis of lung CYP1A1 mRNA expression. The animals were exposed to hyperoxia or hyperoxia + RA, as described under Materials and Methods, and CYP1A1 mRNA expression was analyzed in the lungs by RT-PCR, followed by PCR-Southern analyses at the indicated time points. CYC primers were used as internal control.

Figure 7. Effect of hyperoxia and RA on liver EROD (CYP1A1) activities. Male newborn Fisher rats were treated with hyperoxia and/or RA as described under Materials and Methods, and hepatic EROD (CYP1A1) activities were determined in the microsomes at the indicated time points. Values represent means ± SE (n =4). Two-way ANOVA, followed by modified t-tests

were used to assess statistically significance between individual groups. [a,b,c,d] denote significant differences from CO + air (a), CO + hyperoxia (b), RA + air (c), and RA + hyperoxia (d) at $P < 0.05$.

Figure 8. Effect of hyperoxia and RA on liver MROD (CYP1A2) activities. Male newborn Fisher rats were treated with hyperoxia and/or RA as described under Materials and Methods, and hepatic MROD (CYP1A1) activities were determined in the microsomes at the indicated time points. Values represent means ± SE (n =4). Two-way ANOVA, followed by modified t-tests were used to assess statistically significance between individual groups. [a,b,c,d] denote significant differences from CO + air (a), CO + hyperoxia (b), RA + air (c), and RA + hyperoxia (d) at $P < 0.05$.

Figure 9. Representative western blot showing the effect of hyperoxia and RA on liver CYP1A1/1A2 apoproteins. Rats were treated with hyperoxia and/or RA as described under Materials and Methods, and CYP1A1/1A2 apoprotein expression was determined in liver microsomes (5 μg) of these samples by Western blotting at the indicated time points.

Figure 10. RT-PCR analysis of liver CYP1A1 mRNA expression. The animals were exposed to hyperoxia or hyperoxia + RA, as described under Materials and Methods, and CYP1A1 mRNA expression was analyzed in the livers by RT-PCR, followed by PCR-Southern analyses at the indicated time points. CYC primers were used as internal control.

Figure 11. RT-PCR analysis of liver CYP1A2 mRNA expression. The animals were exposed to hyperoxia or hyperoxia + RA, as described under Materials and Methods, and hepatic CYP1A2 mRNA expression was analyzed by RT-PCR, followed by PCR Southern analyses at the indicated time points. CYC primers were used as internal control.

Figure 12. Scheme showing possible protective mechanisms of RA action on lung injury. We postulate that hyperoxia-mediated induction of CYP1A enzymes could lead to increased ROS production, resulting in lung injury. RA may prevent oxygen-induced lung injury by attenuating CYP1A expression.

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TABLE 1

Quantitation of pulmonary CYP1A1 mRNA levels

Treatment	Age (PND)	Pixel Density ratio of CYP1A1/CYC
CO + Air	8	0.58 ± 0.06
CO + O_2	8	0.78 ± 0.08[a,c,d]
RA + Air	8	0.51 ± 0.04[b]
RA + O_2	8	0.55 ± 0.05[b]
CO + Air	15	0.68 ± 0.06
CO + O_2	15	0.80 ± 0.08[a,c,d]
RA + Air	15	0.61 ± 0.07[b]
RA + O_2	15	0.65 ± 0.05[b]
CO + Air	22	0.65 ± 0.06
CO + O_2	22	0.82 ± 0.08[a,c,d]
RA + Air	22	0.64 ± 0.07[b]
RA + O_2	22	0.60 ± 0.06[b]
CO + Air	38	0.62 ± 0.08
CO + O_2	38	0.91 ± 0.1[a,c,d]
RA + Air	38	0.57 ± 0.04[b]
RA + O_2	38	0.64± 0.07[b]

PCR-Southern blots blots, obtained from experiments described in Materials and Methods, were subjected to phosphor imaging analyses, and the CYP1A1 mRNA levels were estimated in individual animals. Data represent means ± SE of the ratios of pixel densities of CYP1A1 normalized to CYC controls from at least 3 individual animals. Two-way ANOVA, followed by modified t-tests were used to assess statistical significance between individual groups. Different at $P < 0.05$ from CO + air (a), CO + hyperoxia (b), RA + air (c), and RA + hyperoxia (d).

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TABLE 2

Quantitation of hepatic CYP1A1/1A2 mRNA levels

Treatment	Age (PND)	Pixel Density ratio of CYP1A1/CYC	Pixel Density ratio of CYP1A2/CYC
CO + Air	8	1.77 ± 0.23	0.25 ± 0.04
CO + O_2	8	2.2 ± 0.12[a]	0.42 ± 0.05[a,c,d]
RA + Air	8	1.97 ± 0.25	0.31 ± 0.04[a,b,d]
RA + O_2	8	2.05 ± 0.23	0.14 ± 0.02[a,b,c]
CO + Air	15	1.85 ± 0.33	0.85 ± 0.14
CO + O_2	15	2.31 ± 0.14[a,c]	1.31 ± 0.11[a,c,d]
RA + Air	15	1.94 ± 0.28[b]	0.91 ± 0.14[b]
RA + O_2	15	2.08 ± 0.27	0.87 ± 0.09[b]
CO + Air	22	1.88 ± 0.28	1.75 ± 0.24
CO + O_2	22	2.71 ± 0.32[a,c,d]	2.42 ± 0.32[a,c,d]
RA + Air	22	1.91 ± 0.35[b]	1.83 ± 0.21[b]
RA + O_2	22	2.15 ± 0.33[b]	1.61 ± 0.26[b]
CO + Air	38	1.95 ± 0.21	2.35 ± 0.15
CO + O_2	38	3.87 ± 0.35[a,c,d]	3.51 ± 0.31[a,c,d]
RA + Air	38	1.85 ± 0.20[d]	2.10 ± 0.23[b]
RA + O_2	38	2.4± 0.45[a,b,c]	1.82± 0.0.2[a,b]

PCR-Southern blots blots, obtained from experiments described in Materials and Methods, were subjected to phosphor imaging analyses, and hepatic CYP1A1 and 1A2 mRNA levels were estimated in individual animals. Data represent means ± SE of the ratios of pixel densities of CYP1A1 or CYP1A2 normalized to CYC controls from at least 3 individual animals. Two-way ANOVA, followed by modified t-tests were used to assess statistical significance between individual groups. Different at $P < 0.05$ from CO + air (a), CO + hyperoxia (b), RA + air (c), and RA + hyperoxia (d).

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Figure 1

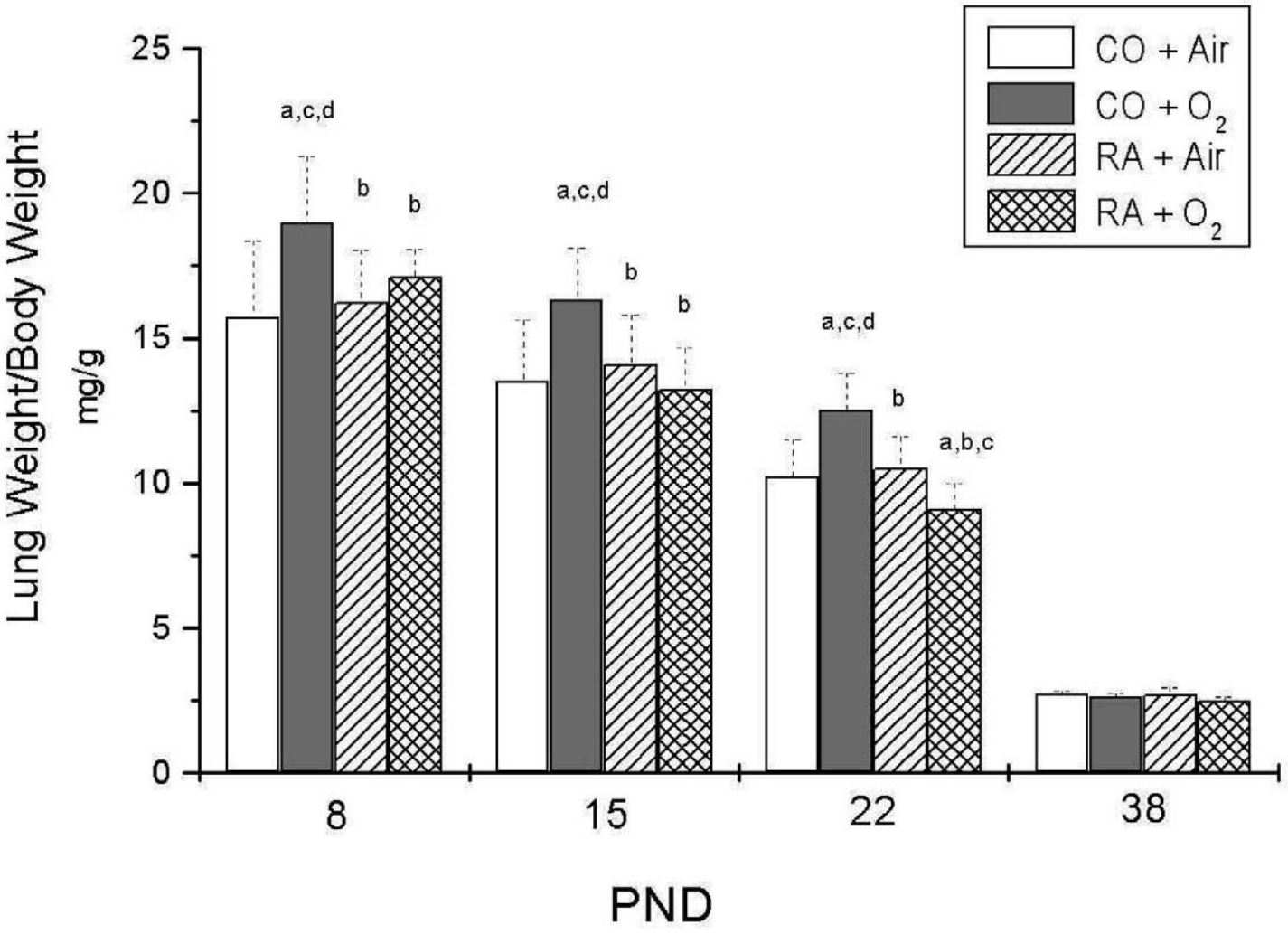

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Figure 2

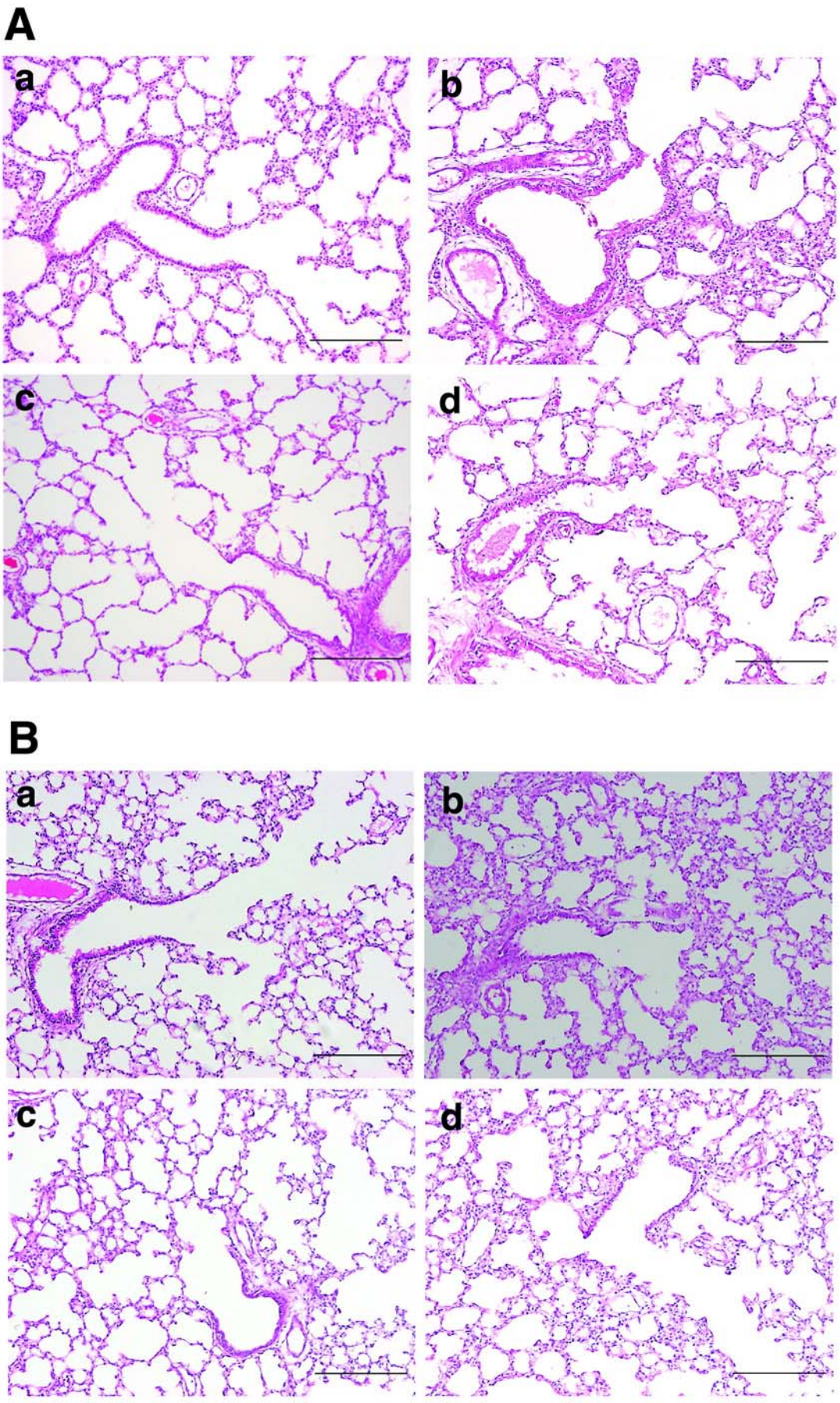

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Figure 3

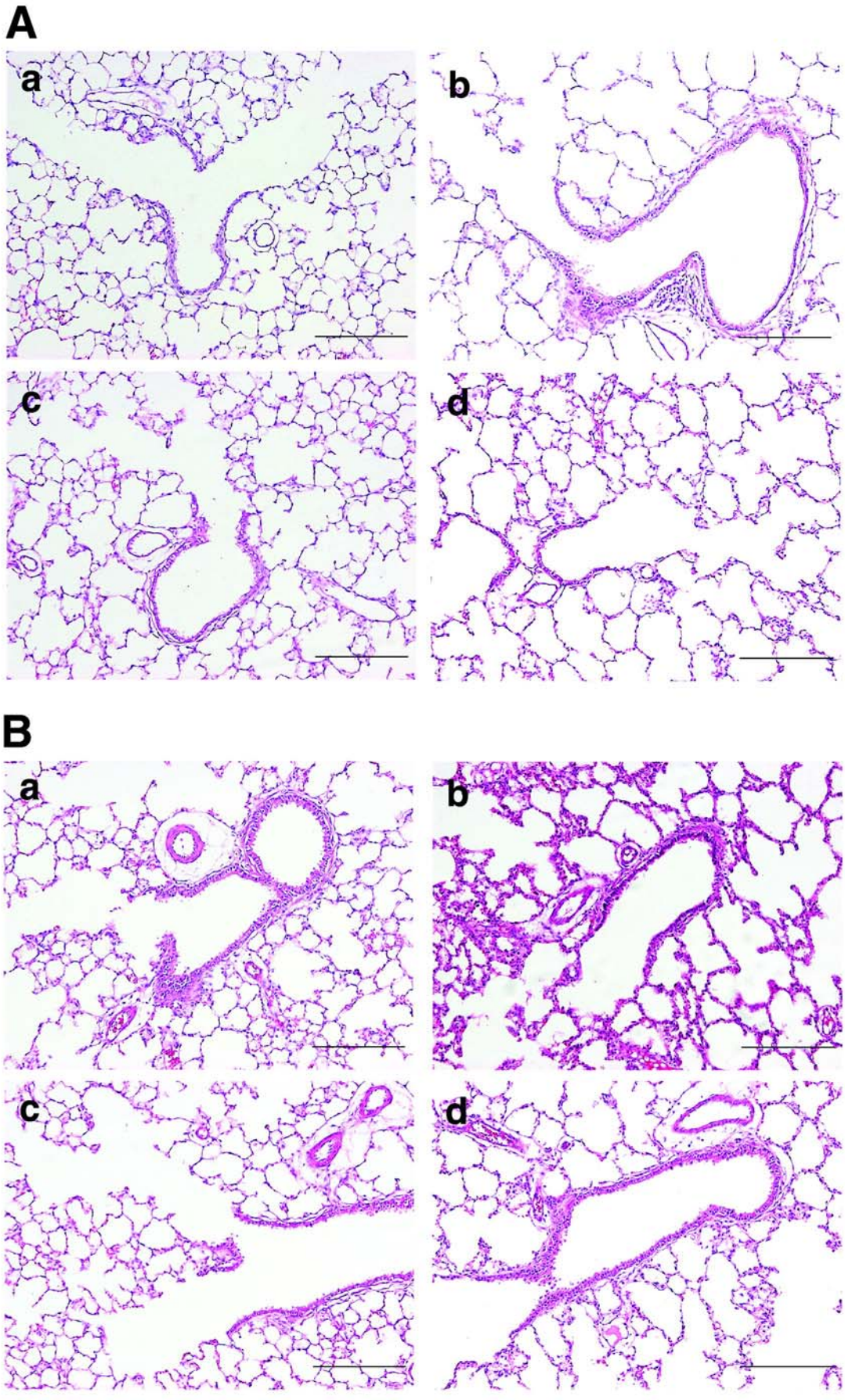

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Figure 4

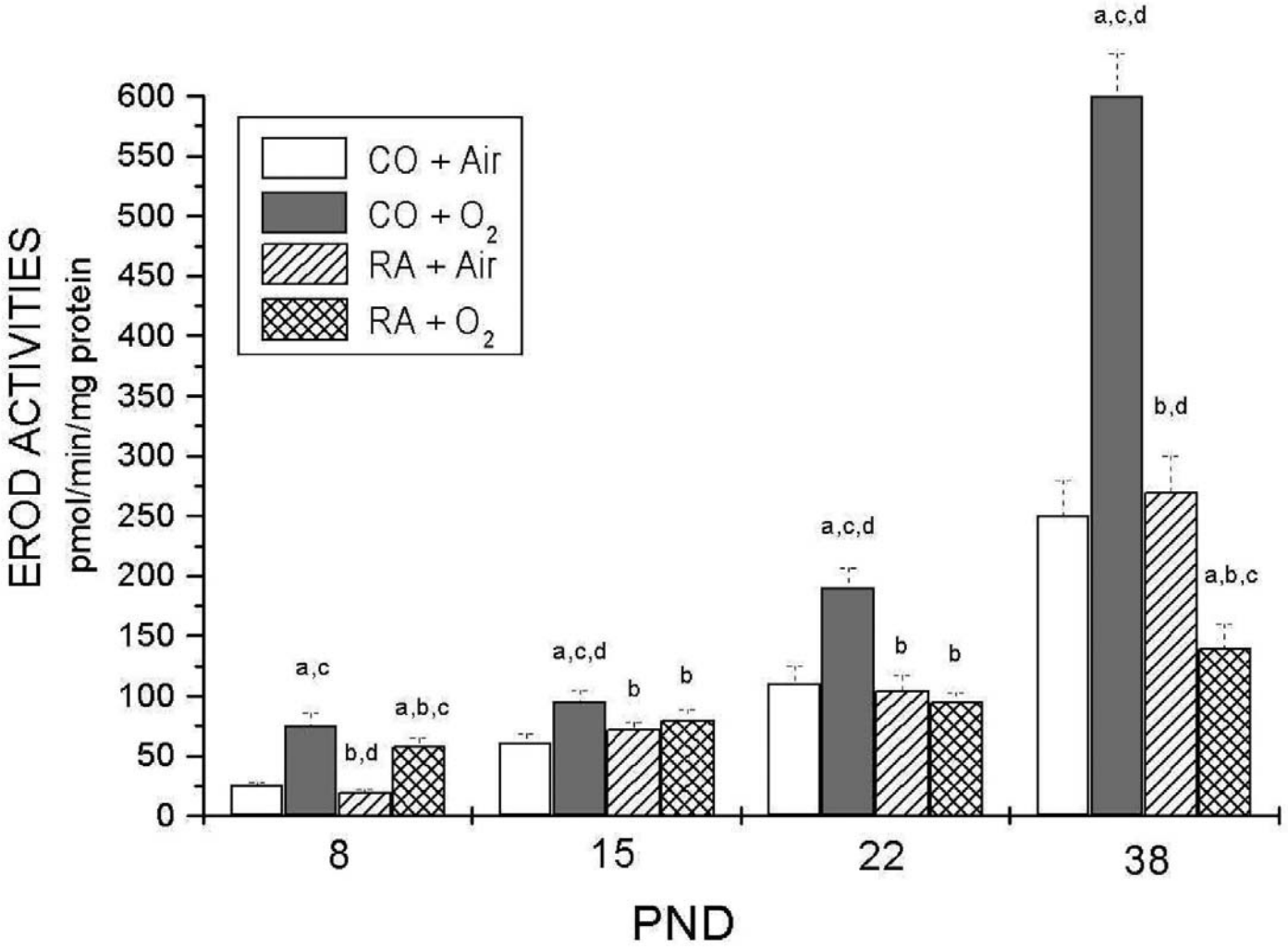

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Figure 5

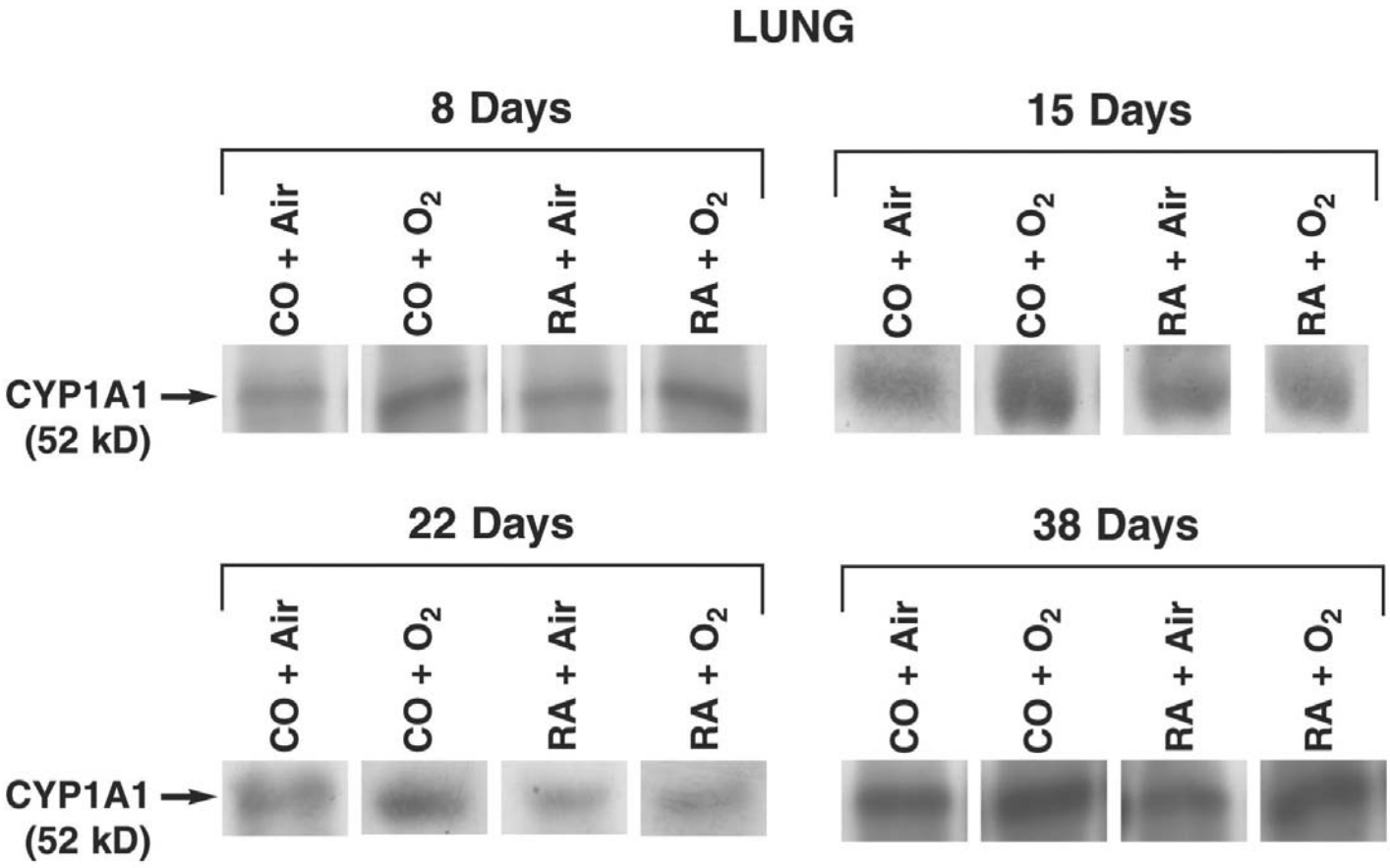

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Figure 6

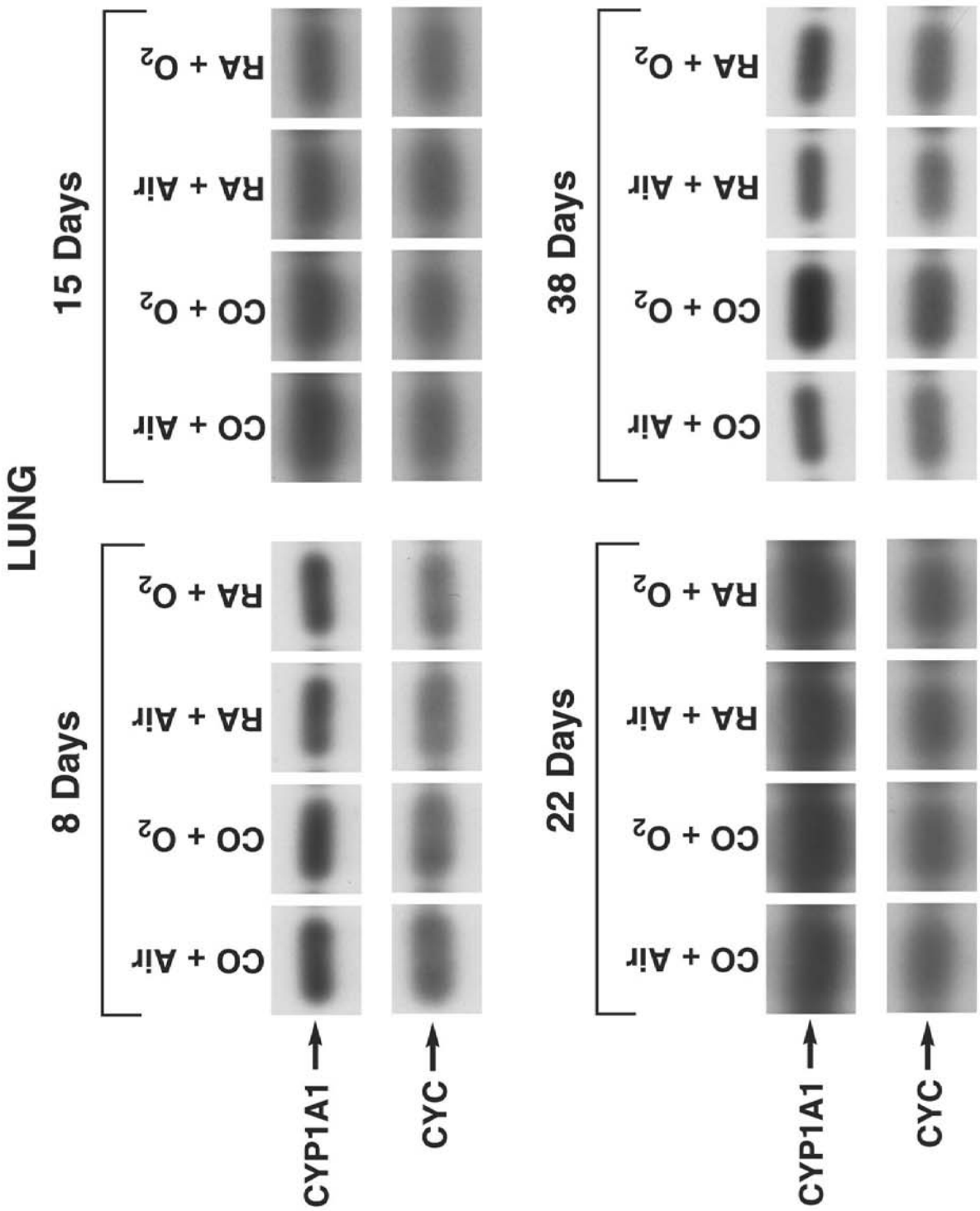

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Figure 7

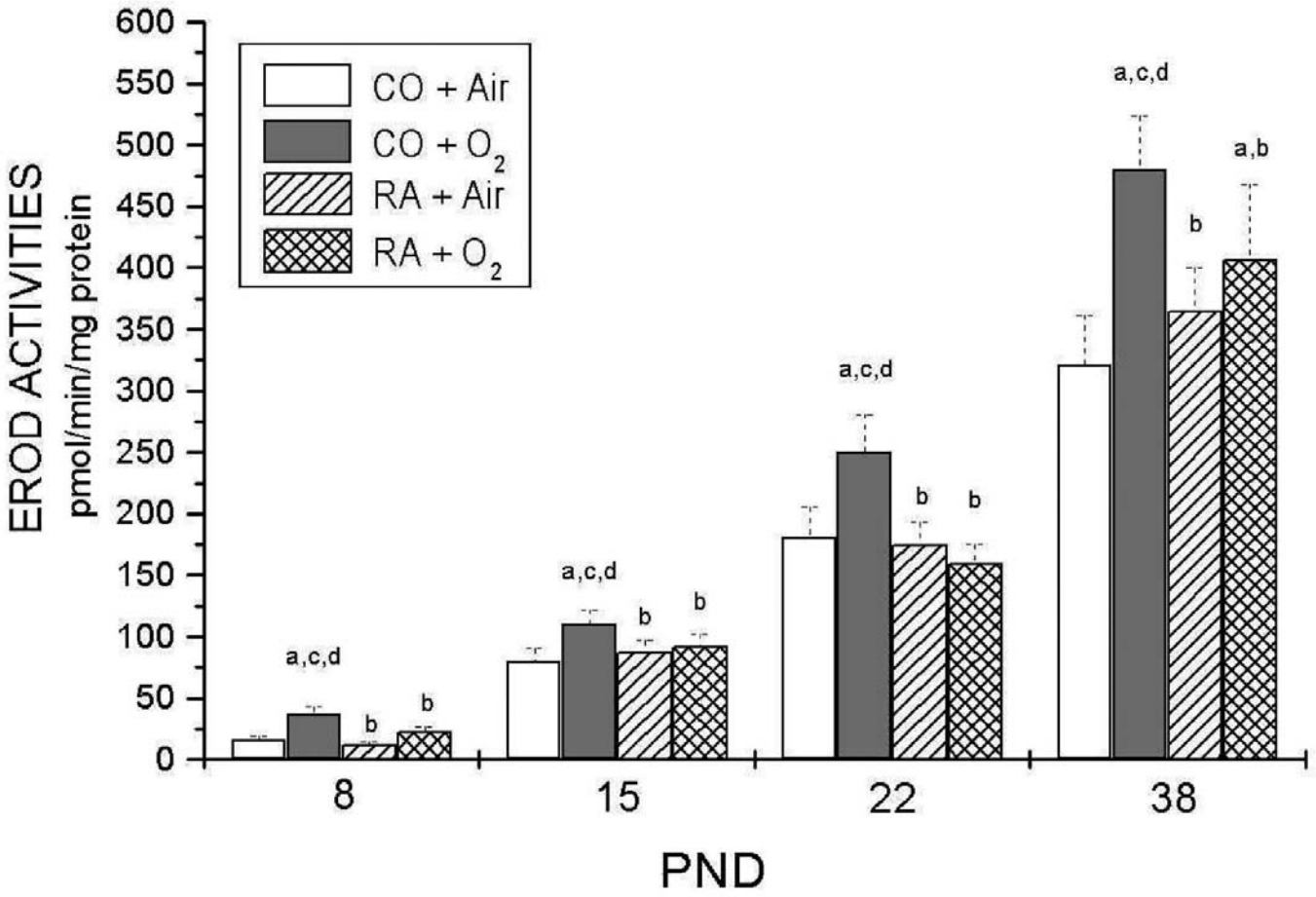

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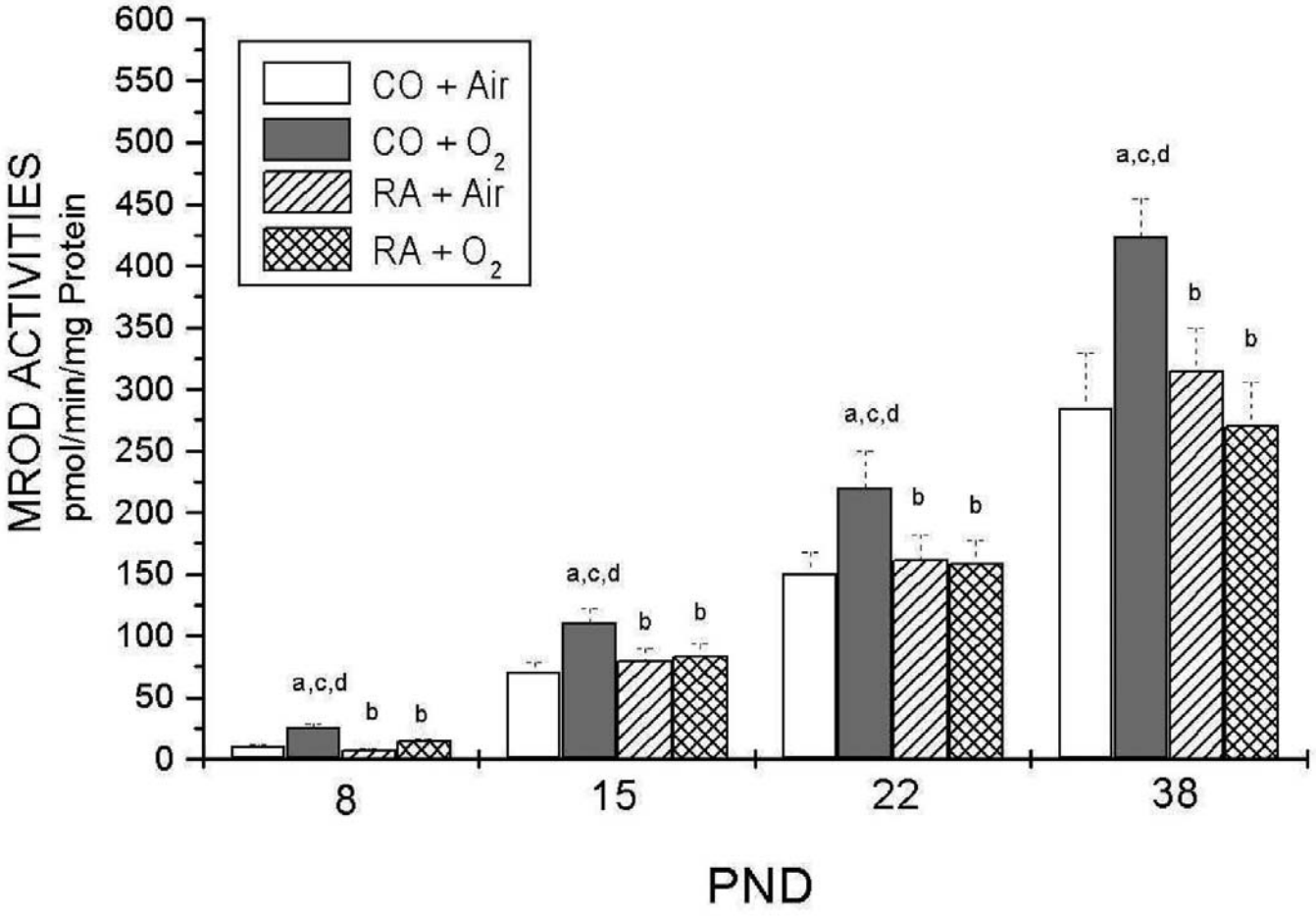
Figure 8



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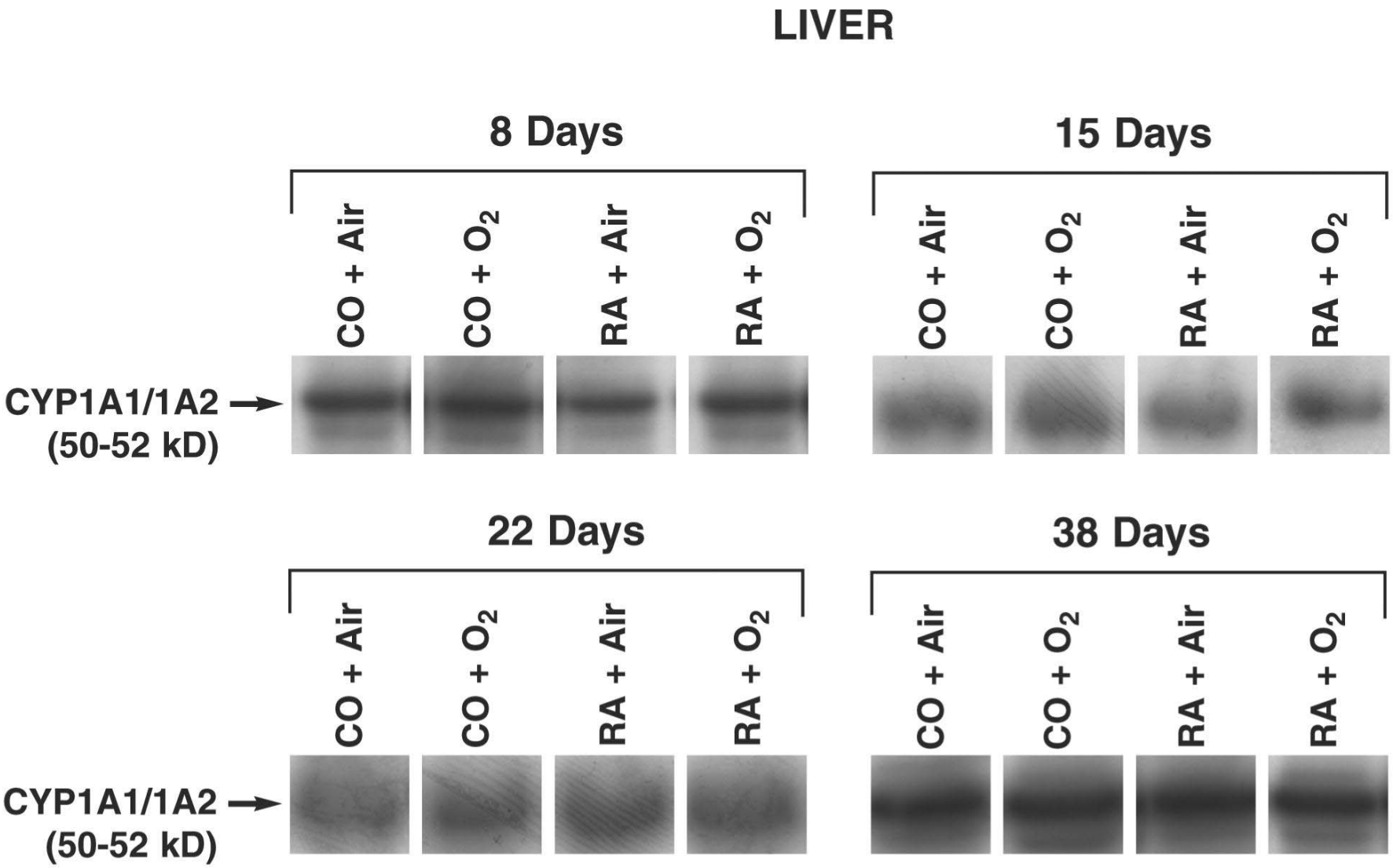

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Figure 10

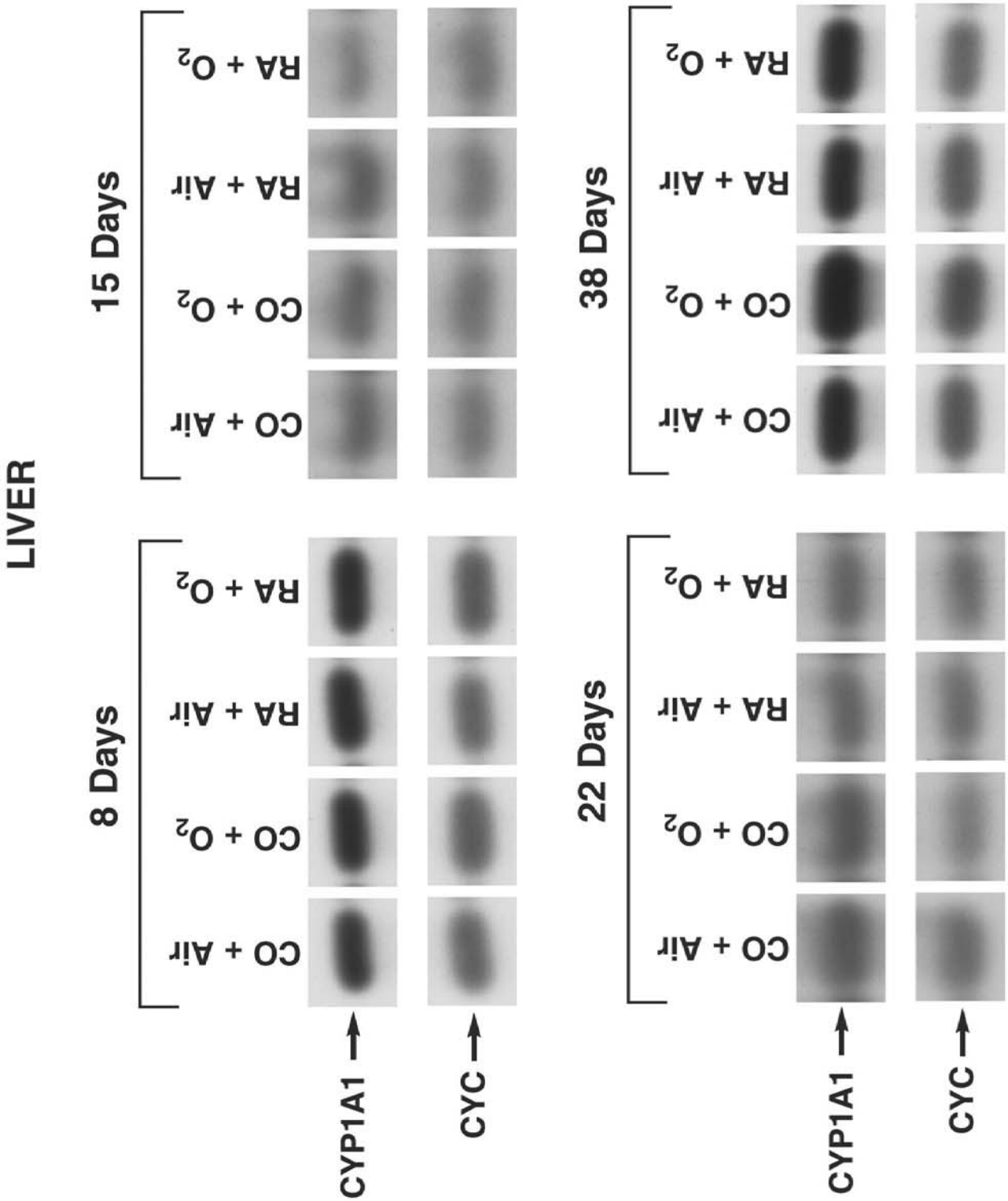

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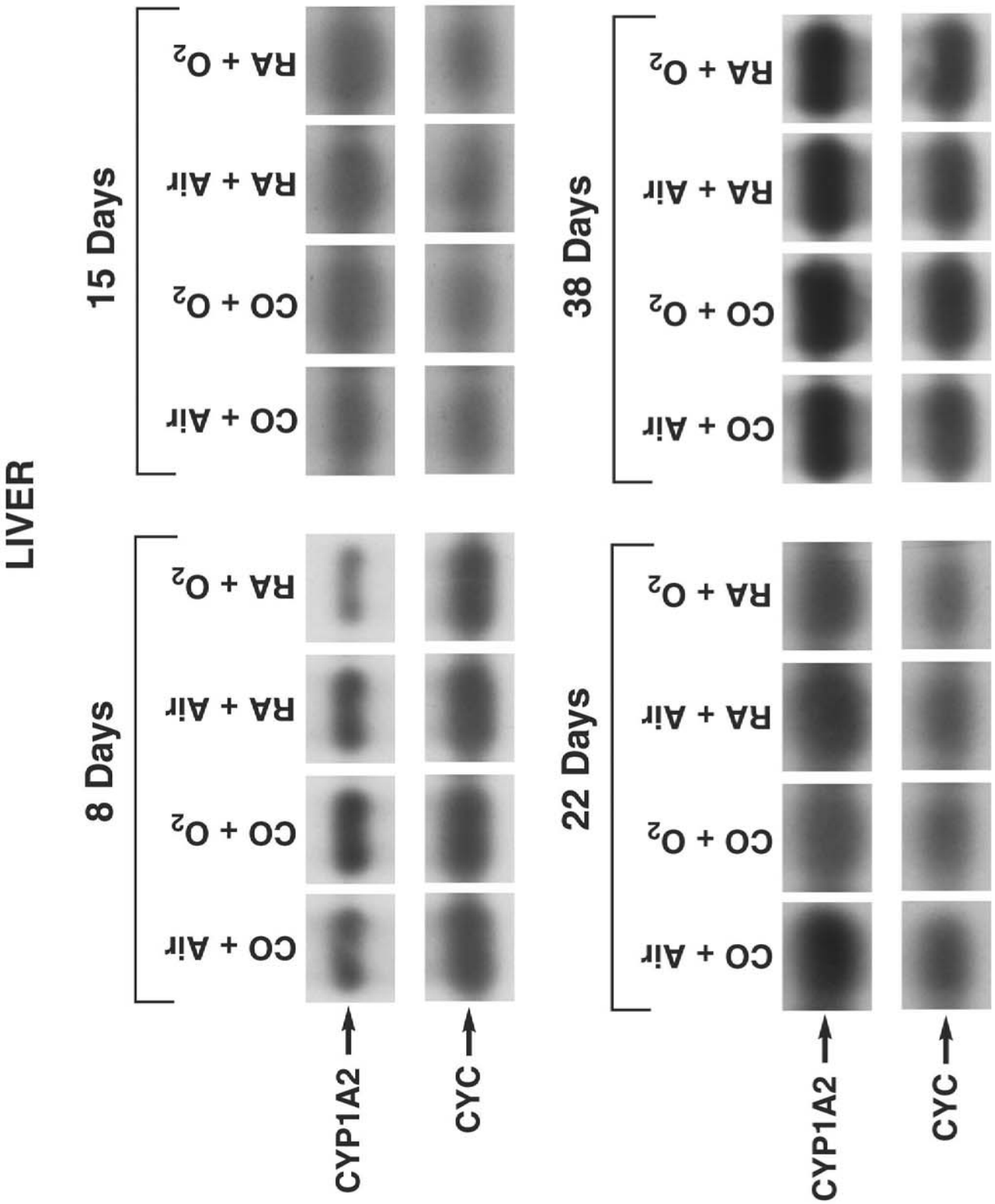
Figure 11



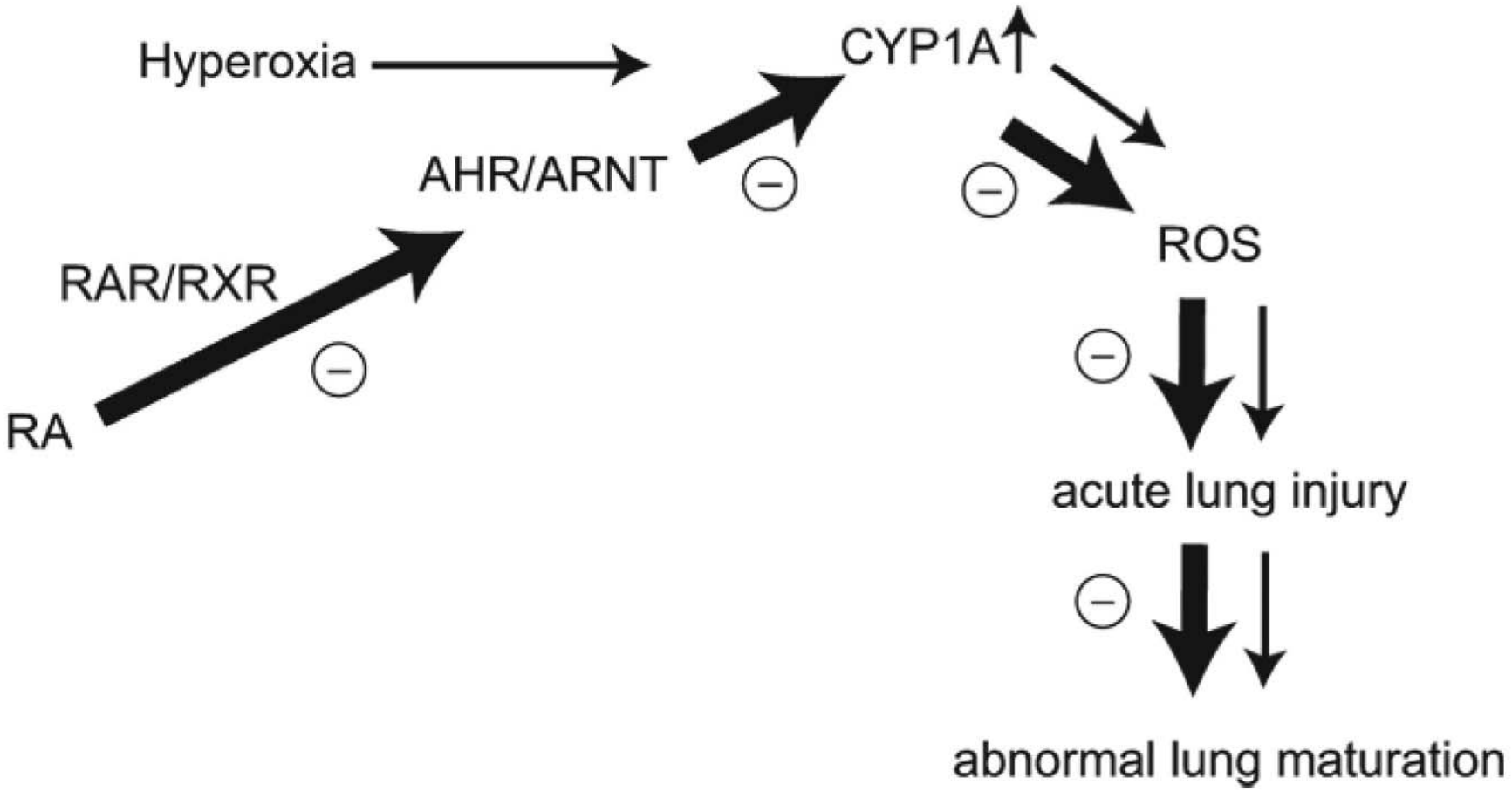


Figure 12