0026-895X/02/6103-507–515$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 61:507–515, 2002

Vol. 61, No. 3
1311/966219
Printed in U.S.A.

Regulation of Pulmonary and Hepatic Cytochrome P4501A Expression in the Rat by Hyperoxia: Implications for Hyperoxic Lung Injury

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Received August 23, 2001; accepted November 28, 2001

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Supplemental oxygen therapy is frequently used in the treatment of pulmonary insufficiency, as is encountered in premature infants, and in patients with acute respiratory distress syndrome. However, hyperoxia causes lung damage in experimental animals and may do so in humans. Cytochrome P4501A enzymes have been implicated in hyperoxic lung injury. In this study, we investigated the mechanisms of CYP1A1 regulation by hyperoxia and tested the hypothesis that aryl hydrocarbon receptor (AHR)-dependent mechanisms contribute to induction of CYP1A1 and that modulation of CYP1A by hyperoxia may have implications for lung injury. Exposure of adult male Sprague-Dawley rats to hyperoxia for 24 to 48 h led to increased expression of pulmonary CYP1A1 enzyme, which was preceded by enhancement of the corresponding mRNA, followed by decline of induction at 60 h, when the animals displayed severe respiratory distress and lung inflammation. Similarly, hepatic CYP1A1/1A2 mRNAs were markedly induced between 24 and 48 h of hyperoxia, with induction declining by 60 h. Electrophoretic mobility shift assays (EMSA) and experiments with AHR (−/−) mice indicated that AHR-dependent mechanisms contributed to CYP1A induction. The AHR (−/−) mice were refractory to CYP1A1 induction by hyperoxia and were more sensitive to lung injury than wild-type mice. Lungs of hyperoxic rats showed increase in the expression of CYP1A1 in airway epithelial cells, type II pneumocytes, and endothelial cells. In conclusion, our results suggest that induction of CYP1A1 by hyperoxia is mediated by AHR-dependent mechanisms and that modulation of CYP1A enzymes by hyperoxia may have implications for hyperoxic lung injury.

Supplemental oxygen therapy is often necessary to sustain life and is frequently employed in the treatment of pulmonary insufficiency, as is encountered in preterm and term infants and in patients with acute respiratory distress syndrome (Northway and Rosan, 1968; Fisher, 1980). However, hyperoxic therapy may contribute to tissue damage and the development of lung diseases, such as bronchopulmonary dysplasia (Northway and Rosan, 1968; Smith and Welty, 1999) and retinopathy of prematurity in preterm infants (Smith and Welty, 1999). Exposure of experimental animals to hyperoxia causes lung damage (Clark and Lambersten, 1971). The mechanisms of hyperoxic lung injury are not completely understood, but most probably involve reactive oxygen species (ROS) (e.g., superoxide anion, hydrogen peroxide, and hydroxyl radical), which may be generated excessively during hyperoxic exposures (Kehrer and Smith, 1994; Yang et al., 1999).

Cytochrome P450 (P450) enzymes belong to a superfamily of hemeproteins that play important roles in the metabolism of exogenous and endogenous chemicals (Nebert and Gonzalez, 1987; Guengerich, 1990). P450 enzymes, including CYP1A1, have also been implicated in the formation and further reactions of ROS (Mansour et al., 1988; Gram, 1997; Morel and Barouki, 1998; Morel et al., 1999, 2000). Several studies have suggested that CYP1A enzymes, which are induced by hyperoxia in rodents, play a role in pulmonary oxygen toxicity (Gonder et al., 1985; Hazinski et al., 1989, 1995; Okamoto et al., 1993; Moorthy et al., 1997; Moorthy, 2000). The mechanisms of induction of CYP1A by hyperoxia have not been determined, al-

This work was supported in part by National Institute of Environmental Health Sciences grant R01-ES09132), a Research Grant from the American Lung Association of Texas, and a Career Investigator Award CL-005-N from the American Lung Association (to B.M.).

ABBREVIATIONS: ROS, reactive oxygen species; P450, cytochrome P450; AHR, aryl hydrocarbon receptor; ABT, aminobenzotriazole; PCR, polymerase chain reaction; CCSP, Clara cell secretory protein; EROD, ethoxyresourufin *O*-deethylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT, reverse transcriptase; CYC, cyclophilin; EMSA, electrophoretic mobility shift assay; AHREs, aryl hydrocarbon response elements; ARNT, aryl hydrocarbon receptor nuclear translocator; MPO, myeloperoxidase; ANOVA, analyses of variance; MC, 3-methylcholanthrene; NF1, nuclear factor 1.

though involvement of the Ah receptor (AHR) has been suggested (Okamoto et al., 1993).

Gonder et al. (1985) showed that *Ah*-responsive C3H/HeJ mice, which display induction of P450 contents by hyperoxia, are more susceptible to hyperoxic lung injury than *Ah*-nonresponsive DBA/2J mice, which do not show P450 induction by hyperoxia. On the other hand, Hudak et al. (1993) demonstrated the C57BL/6J mice to be much more susceptible to oxygen injury than C3H mice, although both these mouse strains are *Ah*-responsive, and Mansour et al. (1988) showed C57BL/6J mice and DBA/2J mice to be equally susceptible to lung injury. Taken together, it seems that susceptibility to hyperoxic injury does not always positively correlate with *Ah*-responsiveness of the animals, and that other factors (e.g., genetic background, diet, antioxidant enzymes) need to be considered. We recently observed that C57BL/6J mice lacking the gene for the liver-specific CYP1A2, which is a member of the *Ah* gene battery, were more sensitive to hyperoxic lung injury than wild-type mice, suggesting that extrapulmonary organs play a role in lung damage (Moorthy et al., 1999). Interestingly, the levels of constitutive CYP1A2 expression are comparable among the C57BL/6J, C3H/HeJ, and DBA/2J mouse strains (Sakuma et al., 1999), suggesting that differential modulation by hyperoxia of CYP1A2 and/or other mechanisms may contribute to the differences in sensitivities to oxygen injury among these mouse strains.

When P450 activities are inhibited in rats with interferon inducers (Kikkawa et al., 1984) or lambs with cimetidine (Hazinski et al., 1989), hyperoxic lung injury is attenuated in these animals. However, the P450 inhibitor aminobenzotriazole (ABT) severely potentiates lung damage by hyperoxia in rats (Moorthy, 2000). Species differences and/or specificities of inhibitors toward different P450 enzymes may explain the observed discrepancies. Whereas ABT inhibits CYP1A1/1A2 and CYP2B1/2B2 activities, cimetidine inactivates CYP2A6 and CYP2C11 but not CYP1A1/1A2, CYP2B1, or CYP3A1/1A2 (Levine et al., 1998). The mechanisms of action of these inhibitors may also contribute to modulation of lung injury. Cimetidine inhibits P450 by interacting with iron (Rendic et al., 1983) and may ameliorate injury by acting as a radical scavenger (Paller and Jacob, 1994). On the other hand, ABT is a mechanism-based inactivator of P450 (Mathews and Bend, 1986), and iron released as result of heme destruction could augment lung damage through free radical reactions (Yang et al., 1999; Moorthy, 2000).

The apparent discrepancies regarding the effects of modulators of P450 on hyperoxic lung injury in different experimental systems warrant further study. We reported recently that exposure of adult rats to hyperoxia for up to 48 h resulted in induction of CYP1A1 and 1A2 in liver, followed by decline at 60 h (Moorthy et al., 1997). However, the mechanisms of regulation of pulmonary and hepatic CYP1A by hyperoxia have not been determined. In this study, we investigated the mechanisms of CYP1A1 regulation by hyperoxia and tested the hypotheses that AHR-dependent mechanisms contribute to induction of CYP1A1 and that modulation of CYP1A by hyperoxia may have implications for hyperoxic lung injury.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (2 months old) were obtained from Harlan Sprague-Dawley (Houston, TX). The animals were acclimatized for 7 days before the studies and were either maintained in room air or exposed to >95% O_2 for 24, 48, or 60 h using pure O_2 at 5 l/min, as we have described previously (Ramsay et al., 1998). Purified tap water and food [Purina Rodent Lab Chow 5001; Purina Mills, Inc., (Richmond, IN)] were available ad libitum. At the termination of their respective exposures, eight rats from each group were anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and euthanized by exsanguination while under deep pentobarbital anesthesia. In four rats from each group, 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 dissected for RNA isolation. In each of the remaining four animals from each group, the left lungs were inflated through the intratracheal catheter and were fixed at constant pressure (20 cm of H_2O) with zinc formalin, after which the lungs were embedded in paraffin for subsequent histological and immunohistochemical analyses (Ramsay et al., 1998). The right lungs were used for RNA isolation.

A breeding pair of AHR (+/−) mice, which were on C57BL/6J background, were obtained from Jackson laboratories (Bar Harbor, ME). Wild-type [AHR (+/+)] and AHR (−/−) mutant mice were obtained by heterozygous (+/−) matings. Animal genotyping was carried out by PCR analysis of tail DNA (Schmidt et al., 1993). Two-month-old adult AHR (+/+) or (−/−) male mice were maintained in room air or exposed to hyperoxia for 48 h. Lung weight/body weight ratios were determined and CYP1A1-dependent enzyme activities were measured in lung microsomes of air-breathing and hyperoxic animals.

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 (Hercules, CA). The primary monoclonal antibody to CYP1A1, which cross-reacts with CYP1A2, was a generous gift from Dr. P. E. Thomas. Clara cell secretory protein (CCSP) antibody was kindly provided by Dr. F. Demayo. Goat anti-mouse IgG conjugated with horseradish peroxidase was from Bio-Rad. CYP1A1 cDNA, which cross-reacts with CYP1A2, was a generous gift from Dr. Frank Gonzalez (National Cancer Institute, Bethesda, MD). GAPDH cDNA was a gift from Dr. Toshiya Tamura of our department. Reverse transcriptase from avian myeloblastosis virus, RNAsin, and dNTPS were from Promega (Madison, WI). *Taq* polymerase was from Invitrogen (Carlsbad, CA).

Preparation of Microsomes and Enzyme Assays. Lungs were perfused with ice-cold phosphate-buffered saline, pH 7.4. Lung microsomes were prepared by differential centrifugation, as reported previously (Moorthy, 2000), from individual animals. Protein concentrations were estimated by the Bradford dye-binding method (Bradford, 1976). Ethoxyresorufin *O*-deethylase (EROD) (CYP1A1) activities in lung microsomes were assayed as we have described previously (Moorthy et al., 1997; Moorthy, 2000).

Electrophoresis and Western Blotting. Lung microsomes (30 μg of protein) prepared from individual animals were subjected to SDS polyacrylamide gel electrophoresis in 7.5% acrylamide gels. The separated proteins on the gels were either stained with Coomassie Brilliant blue dye or were transferred to polyvinylidene difluoride membranes, followed by Western blotting (Moorthy et al., 1997; Moorthy, 2000). Apoprotein levels were estimated by scanning the negatives of the Western blots with laser densitometry, as described previously (Moorthy, 2000). The rationale for using 30 μg of protein was based on pilot experiments showing that this amount of protein yielded CYP1A1 immunostaining intensity that was in the linear range.

Northern Blotting. Total liver or lung RNA was isolated from individual animals using a modification of the procedure of Chomczynski and Sacchi (1987). RNA (20 μg/per sample) was loaded onto 1% agarose/formaldehyde denaturing gel, separated by electrophoresis, and transferred to nitrocellulose filters. Northern hybridization was performed by using random prime ^{32}P-labeled labeled CYP1A1 cDNA probe (20×10^6 cpm) (Moorthy, 2000). After autoradiography of the hybridized membranes, the membranes were stripped by several washes and re-probed with random prime labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. Relative levels of CYP1A1/1A2 mRNAs were quantitated by filmless autoradiographic system analysis. GAPDH cDNA probe was used as an internal control to assess RNA transfer, loading, and hybridization.

RT-PCR Assays. Total RNA (20 μg) from livers of air-breathing and hyperoxic animals was reverse-transcribed (Wang and Strobel, 1997), and the resulting cDNA was used as template for PCR analysis. Primers specific for CYP1A1 (5′ GATGCTGAGGACCAGAAGACCGC 3′ and 5′ CAGGAGGCTGGACGAGAATGC 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 (Geng and Strobel, 1998). CYC gene was amplified in the same tube as CYP1A1.

Southern Blot Analysis of PCR Products. The PCR products, generated by PCR amplification of cDNA for different cycle numbers (18–30 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 CYC, which were prepared by PCR amplification, followed by purification and extraction of the PCR products from agarose gels (Wang and Strobel, 1997). The membranes were exposed to a phosphor screen, and pixel densities of the PCR products were measured. The effect of hyperoxia on the level of lung CYP1A1 mRNA was measured by determining ratios of band intensities of CYP1A1 and CYC.

Electrophoretic Mobility Shift Assay. Nuclei and nuclear protein extracts from livers of individual air-breathing or hyperoxic animals were prepared according to the procedure of Okino et al. (1993). The nuclear protein extracts were stored at −80°C until use. EMSAs were performed as described by Okino et al. (1993). Briefly, the nuclear proteins (15 μg), suspended in EMSA buffer (25 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 500 mM KCl, pH 7.5), were preincubated with poly dI-dC (2.5 μg) on ice for 10 min, followed by incubation at room temperature for 20 min with 75,000 cpm of double-stranded oligonucleotide probe, which contains the consensus sequences [aryl hydrocarbon response elements (AHREs)] for AHR/AHR nuclear translocator (ARNT) binding (Okino et al., 1993). The DNA fragments that were used to make the double-stranded oligonucleotide probe were 5′-GATCCGGCTCTTGTCACGCAACTCCGAGCTCA-3′ and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′ (Okino et al., 1993). The oligonucleotide probe was prepared by annealing the single-stranded fragments, followed by 5′-end labeling of the double stranded DNA fragment with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. For competition experiments, nuclear proteins were incubated with 25-fold excess of unlabeled probe before addition of the labeled probe. Supershift assays were conducted by incubating nuclear proteins with AHR antibody (2 μg) for 2 h on ice, after which the assay was performed as described above. The labeled samples were separated by nondenaturing polyacrylamide gel electrophoresis (4% gels) at 200 V for 2 h. The gels were dried and exposed to autoradiography using Kodak X-ray film (Eastman Kodak, Rochester, NY).

Immunohistochemistry. Immunohistochemistry was performed on tissue sections from individual animals (Ramsay et al., 1998). Paraffin sections were stained with antibody directed against CYP1A1, CCSP, or myeloperoxidase (MPO). Sections were incubated in a 1:75 solution of normal goat serum (for CCSP and MPO analyses) or horse serum (for CYP1A1 analysis) for 20 min at room temperature. After removal of the serum, the respective antibody (CYP1A1, CCSP, or MPO) was applied to the slides. The CYP1A1 antibody concentration was 2 μg/ml. For CCSP and MPO analyses, the antibody dilution factor was 1:20,000 and 1:3,200, respectively. Antibody incubations of slides were for 90 min at room temperature. After incubation, the sections were placed in either a rat adsorbed biotin-conjugated horse anti-mouse IgG (CYP1A1) or biotin-conjugated goat anti-rabbit IgG (CCSP, MPO), which was applied at 2.25 μg/ml for 45 min at room temperature. The sections were treated with a peroxidase tagged avidin-biotin complex for 45 min at room then rinsed well in buffer, and were counter-stained in eosin, dehydrated, rinsed in xylene, and mounted using a synthetic mounting medium.

Statistical Analyses. Data are expressed as means ± S.E. Student's t test, one-way analyses of variance (ANOVA), followed by post hoc Newman-Keul's tests, or two-way ANOVA, followed by modified t tests, were used to assess significant differences arising from exposure to hyperoxia for different time points. P values < 0.05 were considered significant.

Results

Effect of Hyperoxia on Pulmonary CYP1A1 Activities and Contents. Exposure of animals to hyperoxia for 24 h did not alter lung EROD (CYP1A1) activities, compared with those in room air (Fig. 1). However, in animals exposed to 48 h of hyperoxia, the EROD (CYP1A1) activities (Fig. 1) were approximately seven times higher than those of control animals. The induction of EROD activities was followed by a dramatic decline after 60 h of hyperoxia. Hyperoxia-induced augmentation of CYP1A1 enzyme activities was paralleled by enhanced levels of the CYP1A1 apoproteins, as analyzed by Western blotting (Fig. 2). Lung CYP1A1 contents were not altered after 24 h of hyperoxia but were almost doubled after 48 h (not shown). As with the enzyme activities, the apoprotein levels declined by 60 h (Fig. 2).

Lung CYP1A1 mRNA. CYP1A1 mRNA transcripts were not detectable in lungs of air-breathing or hyperoxic animals after hyperoxia, as determined by Northern blotting (not shown). When RNA from lungs of air-breathing and hyperoxic animals was analyzed by RT-PCR, constitutive expression of CYP1A1 was detected (Fig. 3A). The intensity of the 660-base pair PCR product, which corresponded to CYP1A1,

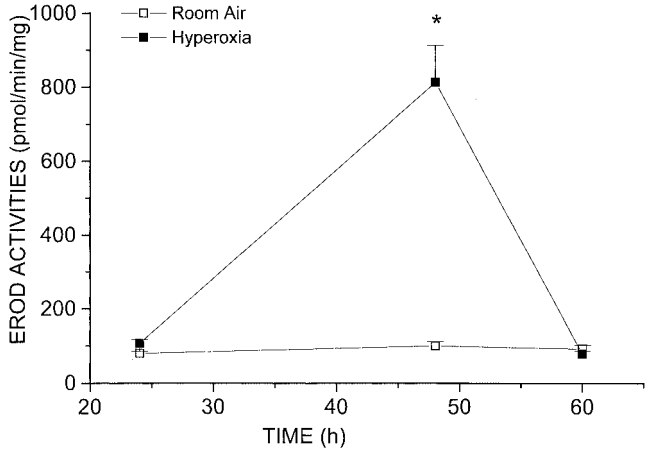

Fig. 1. Effects of hyperoxia on lung microsomal EROD activities. Male Sprague-Dawley rats were maintained in room air or exposed to hyperoxia for 24, 48, or 60 h, and EROD activities were measured in the lung microsomes. Values represent means ± S. E. (n = 4). *, different from air-breathing animals and from animals exposed to hyperoxia for 24 or 60 h, as determined by two-way ANOVA.

was increased 24 h after hyperoxia exposure (Fig. 3A). The mRNA was no longer detectable after prolonged hyperoxia for 48 or 60 h (Fig. 3A), and this was the case even when CYC primers were omitted in the PCR reactions (not shown). No apparent changes were observed in the expression of CYC mRNA from lungs of animals exposed to hyperoxia (Fig. 3A). The effects of hyperoxia on CYP1A1 mRNA were similar when RNA from lungs of individual animals exposed to hyperoxia for a given period of time was analyzed, indicating the reproducibility of the data.

Semiquantitative Analyses of mRNA by PCR-Southern Analysis. To quantify the level of specific CYP1A mRNA in tissues, we used RT-PCR, followed by Southern blotting, as described under *Materials and Methods*. Filmless autoradiographic system analysis of the nylon membranes revealed that PCR amplification for 18 cycles yielded PCR product formation of CYP1A1 and CYC (not shown), which were in the linear range (Fig. 3, B and C). We therefore used 18 PCR cycles to simultaneously amplify CYP1A1 and CYC cDNA, derived from total lung RNA from individual animals that breathed either room air or hyperoxia for 24 h. Hyperoxia for 24 h augmented CYP1A1 but not CYC mRNA (Fig. 3, B and C). A signal corresponding to CYP1A1 mRNA was not detected in room air samples that were subjected to 18 PCR cycles (Fig. 3C, lane 7). These membranes had been exposed to autoradiography for 5 min at room temperature. However, after autoradiographic exposure for 2 h at room temperature, the CYP1A1 signal was clearly visible in the room air samples (not shown). Quantitation of the autoradiograms by filmless autoradiographic system analyses of RT-PCR products from RNA of individual animals revealed that the extent of CYP1A1 mRNA induction by hyperoxia was ~70% (not shown). Similar induction ratios were obtained when quantitative comparisons were made between room air and hyperoxia samples that were subjected to 20 PCR cycles, which also yielded CYP1A1 and CYC products that were in the linear range (not shown).

Effect of Hyperoxia on Hepatic CYP1A1/1A2 mRNA. CYP1A1 mRNA was not detectable in liver by Northern hybridization. However, RT-PCR experiments, using techniques that were similar to those described for lung RNA, showed hepatic CYP1A1 mRNA to be dramatically induced after 48 h of hyperoxia (Fig. 4A). The CYP1A1 mRNA signal disappeared after 60 h of hyperoxia (Fig. 4A). The mRNA levels of hepatic CYP1A2, which is a liver-specific P450 isoform, were approximately doubled after 48 h of hyperoxia, as measured by Northern blotting (Fig. 4B). By 60 h, the CYP1A2 mRNA levels declined to levels that were slightly above room-air–breathing control animals (Fig. 4B), which were in contrast to CYP1A1 mRNA, whose expression was markedly diminished at this time point (Fig. 4A). GAPDH mRNA levels were not altered in animals exposed to hyper-

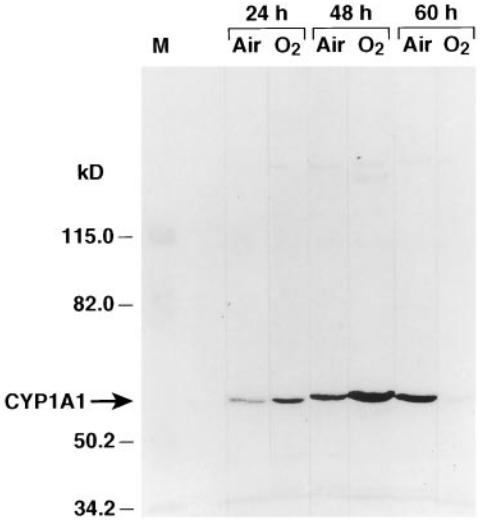

Fig. 2. Representative CYP1A1 apoprotein profile in lungs of animals exposed to hyperoxia. Lung microsomes, isolated from individual animals maintained in room air or exposed to hyperoxia for 24, 48, or 60 h, were subjected to Western blotting using monoclonal antibodies raised against CYP1A1, as described under *Materials and Methods*.

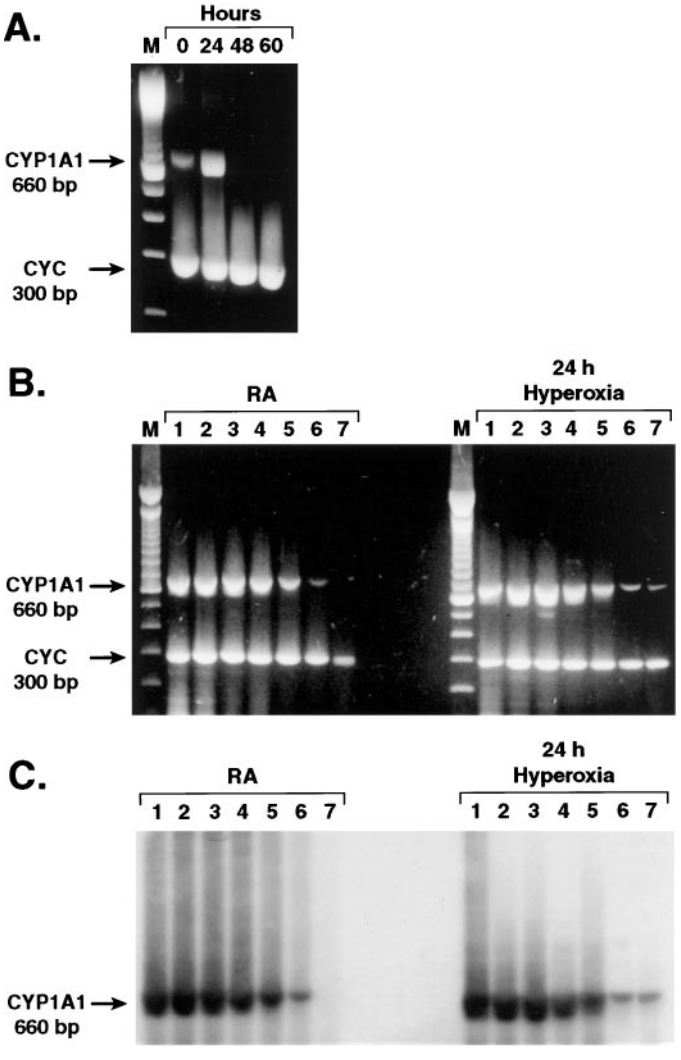

Fig. 3. Representative RT-PCR analysis of lung CYP1A1 mRNA of air-breathing and hyperoxic animals. A, total RNA from lungs of animals breathing room air (RA) (lane 1) or exposed to hyperoxia for 24 (lane 2), 48 (lane 3), or 60 h (lane 4) was reverse-transcribed, and the resulting cDNA was used as template for PCR analysis. Primers specific for CYP1A1 and CYC, were used in PCR reactions (28 cycles) to detect the corresponding genes. B, primers specific for CYP1A1 and CYC were used in multiple PCR reactions, wherein the cycle number was the variable. Lanes 1 to 7 denote PCR reactions performed for 30, 28, 26, 24, 22, 20, and 18 cycles, respectively. C, the PCR products were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes, as described under *Materials and Methods*. The membranes were probed with ^{32}P-labeled cDNA probes specific for CYP1A1 or CYC, and exposed to autoradiography for 5 min at room temperature, followed by quantitation of the signals by filmless autoradiographic system analysis.

oxia (Fig. 4B). Similar effects were observed when several individual animals exposed to hyperoxia were analyzed for CYP1A1/1A2 mRNA expression (data not shown).

Interaction of Nuclear Proteins with AhREs in Hyperoxic Animals. To determine whether the hyperoxia induces CYP1A1/1A2 by AHR-dependent mechanisms, nuclear proteins from livers of room air and hyperoxic animals were incubated with ^{32}P-labeled oligonucleotides that contain the AHREs, which are present in multiple copies on the CYP1A1 promoter (Okey et al., 1994). As shown in Fig. 5, no band shift was observed in air-breathing animals or animals that were exposed to 12 h of hyperoxia. However, exposure of rats to hyperoxia for 24 or 48 h showed a specific band shift, which was competed off in the presence of a 25-fold excess of cold probe (lane 5). Incubation of nuclear proteins with AHR antibody resulted in a supershift of the hyperoxia-specific band. Comparison of EMSA of nuclear proteins isolated from hyperoxic animals with that from MC-treated animals showed that the protein-DNA complex in hyperoxic animals had a lower electrophoretic mobility than that observed in animals that were exposed to MC. A supershift of the MC-specific protein-DNA band was also observed when nuclear proteins from MC-treated animals were incubated with the AHR antibody (not shown). Because of the paucity of nuclear proteins from lungs, EMSA was not performed in the lungs of animals exposed to hyperoxia.

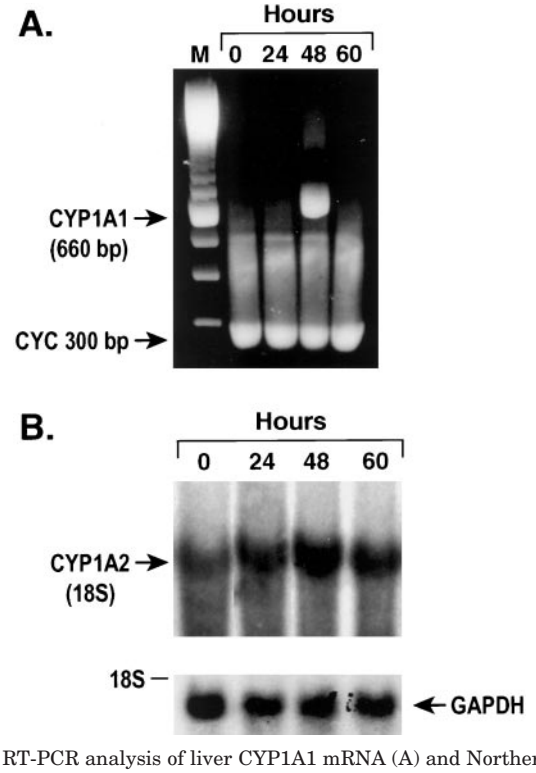

Fig. 4. RT-PCR analysis of liver CYP1A1 mRNA (A) and Northern analysis (B) of liver CYP1A1/1A2 mRNA of air-breathing and hyperoxic animals. A, total RNA from livers of air-breathing and hyperoxic animals was reverse-transcribed, and the resulting cDNA was used as template for PCR analysis. Lanes 1 to 4 represent RNA from animals maintained in room air (lane 1) or exposed to hyperoxia for 24 (lane 2), 48 (Lane 3), or 60 h (lane 4). B, total RNA samples from livers of air breathing and hyperoxic animals were subjected to Northern blotting and CYP1A1/1A2 mRNAs were analyzed by random prime-labeled cDNA probe that recognized both CYP1A1 and 1A2 mRNAs. CYP1A1 mRNA was not detected in any of the samples. The membranes were washed and reprobed with GAPDH cDNA, which was used as an internal control.

Hyperoxia Induces CYP1A1 Expression in Rat Lung in a Cell-Specific Manner. In air-breathing animals, positive staining for CYP1A1 was observed in airway epithelial cells of the bronchiole, but no staining was noticed in endothelial cells (Fig. 6a). However, after 24 (Fig. 6b) and 48 h (Fig. 6c) of hyperoxia, enhanced intensity of staining in airway epithelial cells and endothelial cells was observed. Positive CYP1A1 staining was also observed in cells that were visually identified as alveolar macrophages. At 60 h of hyperoxia, the CYP1A1 staining was diminished in the endothelial cells and alveolar macrophages (Fig. 6d). CYP1A1 staining in the airway epithelial cells seemed to colocalize with CCSP, which is specifically present in Clara cells (Fig. 6e). At higher magnification, staining for CYP1A1 was clearly visible in type II pneumocytes of air-breathing animals (Fig. 7a). In animals exposed to hyperoxia for 24 h, the intensity of CYP1A1 in the type II pneumocytes seemed augmented, and CYP1A1 staining was also observed in alveolar macrophages (Fig. 7b).

Hyperoxia Induces Lung Injury and Inflammation. The effects of hyperoxia on lung inflammation and injury were observed by immunohistochemistry of lung sections using MPO antibody and by routine microscopy. Lungs of air-breathing animals (Fig. 8a) and animals exposed to 24 h of hyperoxia (Fig. 8b) showed only a few MPO-positive neutrophils. However, after 48 (Fig. 8c) and 60 h (Fig. 8d) of hyperoxia, there was massive recruitment of MPO-positive neutrophils into the lungs, and lung injury as evidenced by fluid accumulation in the alveolar spaces.

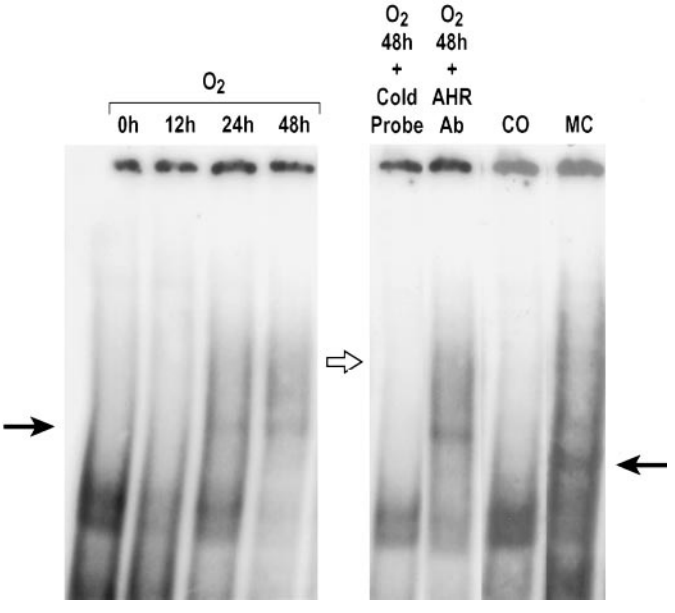

Fig. 5. Representative EMSA of nuclear extracts from room air and hyperoxic rats. Male Sprague-Dawley rats (200 g) were maintained in room air or were exposed to hyperoxia for 12 to 48 h, and animals were sacrificed at different time points. Some animals were treated with the vehicle corn oil (CO) or MC, as positive control. Nuclear protein extracts from livers of these animals were subjected to EMSA, as described under *Materials and Methods*. Some samples (nuclear proteins) were incubated with a 25-fold excess of cold probe or with AHR antibody before EMSA. The labeled nuclear proteins were separated on 4% polyacrylamide gel electrophoresis, and the gels were dried and exposed to autoradiography at −80°C for 16 h. Left arrow indicates interaction of the AHREs with a hyperoxia-specific nuclear protein that seems to be of lower electrophoretic mobility than the MC-AHR-ARNT complex (right arrow). Open arrow, supershifted band in the presence of AHR antibody.

Hyperoxia Fails to Induce CYP1A1-Dependent Activities in AHR (−/−) Mice. AHR (+/+) mice exposed to hyperoxia for 48 h elicited a 50% statistically significant increase in pulmonary EROD (CYP1A1) activities over those of air-breathing animals (Fig. 9). However, AHR (−/−) mice were refractory to induction of CYP1A1 by hyperoxia (Fig. 9). The basal pulmonary EROD activities in air-breathing AHR (−/−) animals were much lower than those of wild-type animals (Fig. 9).

AHR (−/−) Mice Are More Susceptible to Hyperoxic Lung Injury than AHR (+/+) Mice. As shown in Fig. 10, AHR (−/−) animals had significantly higher lung weight/body weight ratios (an index of lung damage) than wild-type mice that were exposed to hyperoxia for 48 h.

Discussion

The marked increases (~7-fold) in lung EROD activities (Fig. 1) and CYP1A1 apoprotein levels (Fig. 2) caused by exposure to hyperoxia for 48 h indicate induction of CYP1A1. The extent of induction of CYP1A1 by hyperoxia was comparable with that mediated by treatment of rats with a single dose (5 μmol/kg) of the prototype CYP1A1 inducer MC in liver (Moorthy, 2000). EROD activities are known to primarily reflect catalytic activities of CYP1A1 (Moorthy et al., 1997; Moorthy, 2000). The observation that pulmonary CYP1A1 apoprotein levels were enhanced in the animals exposed to hyperoxia for 48 h supported the notion that the augmented EROD activities reflected CYP1A1 induction.

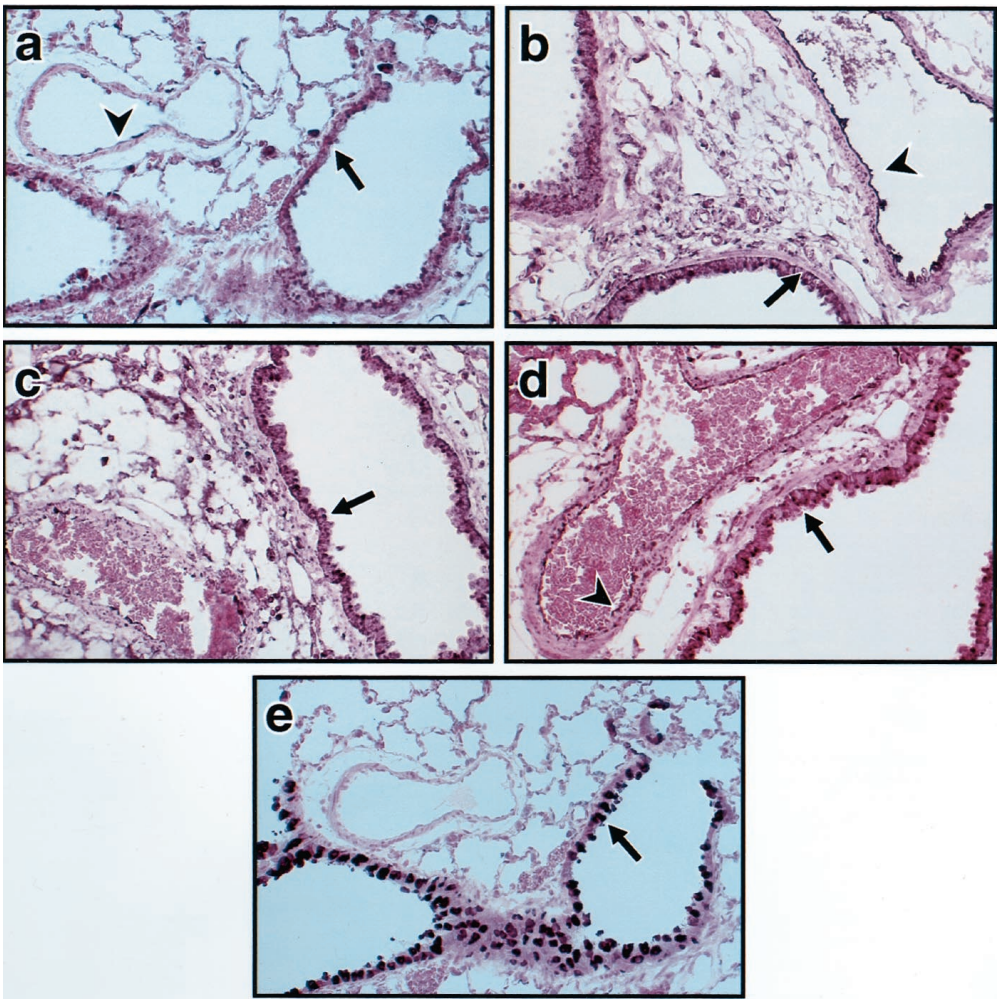

Fig. 6. Representative lung sections for CYP1A1 (a-d) (original magnification, 100×) or CCSP (e) (original magnification, 66×) immunohistochemistry from rats maintained in room air (a, e) or exposed to hyperoxia for 24 (b), 48 (c), or 60 (d) h. Paraffin-embedded tissues were sectioned, mounted, and incubated with CYP1A1 antibody, as described under *Materials and Methods*, to show CYP1A1-staining cells to be purple against a pink eosin counterstain. Arrows point to airway containing epithelial cells of the bronchiole. Air-breathing animals (a) show CYP1A1 positivity in the epithelial cells of the bronchiole and no staining in endothelial cells (arrowhead). e, immunohistochemistry using CCSP antibody. The intense purple stain shows the localization of Clara cells in the airway.

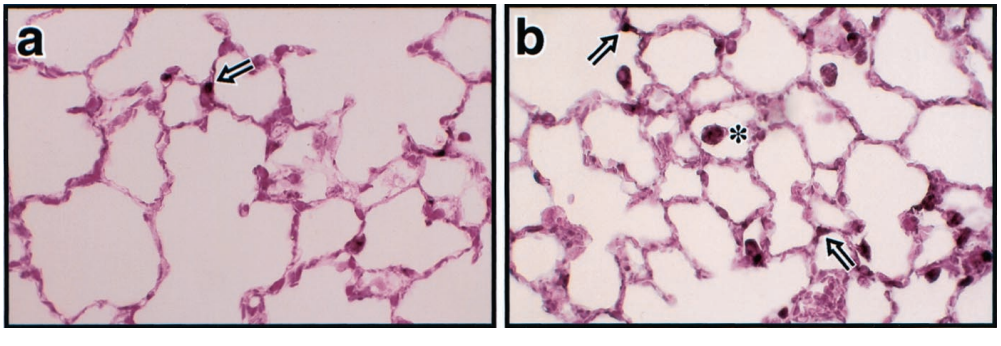

Fig. 7. Representative lung sections (original magnification, 132×) for CYP1A1 immunohistochemistry from rats maintained in room air (a) or exposed to hyperoxia for 24 h (b). Hyperoxic animals show increased CYP1A1 positivity in type II pneumocytes (double arrow) and macrophages (*).

The fact that the increase in CYP1A1 apoprotein expression was lesser than that of EROD activity may have been due to contribution of enzymes other than CYP1A1 (e.g., CYP1B1) that catalyzed EROD activity (Shimada et al., 1997).

The increase in lung CYP1A1 mRNA expression after 24 h of hyperoxia (Fig. 3, A–C) was consistent with the hypothesis that CYP1A1 activities and apoprotein contents were caused, in part, by activation of CYP1A1 gene expression. Hazinski et al. (1995) showed induction of CYP1A1 RNA by hyperoxia in cultured endothelial cells from lambs to be mediated by transcriptional mechanisms. The fact that the expression of CYP1A1 mRNA was markedly suppressed between 24 and 48 h (Fig. 3A) strongly suggests that hyperoxia-induced decline of EROD activities and apoprotein contents was caused by down-regulation of CYP1A1 expression at the pretranslational level. Morel et al. (1999) showed CYP1A1-dependent increases in the formation of H_2O_2 in human hepatoma cells treated with benzo[*a*]pyrene. Because H_2O_2 is known to formed in response to hyperoxia (Freeman and Crapo, 1981), it is possible that CYP1A1-mediated increases in H_2O_2 production may have attenuated CYP1A1 gene expression by an autoregulatory loop mechanism involving down-regulation of NF1, a protein whose binding to the NF1 site on the basal transcription element of the CYP1A1 promoter is critical to the expression of the gene (Morel and Barouki, 1998; Morel et al., 1999). The possibility that H_2O_2 caused CYP1A1 destruction by oxidative degradation of the enzyme itself has not been excluded.

The decrease in the expression of CYP1A1 apoprotein between 48 and 60 h of hyperoxia may also have involved other mechanisms. The half-life of CYP1A1 protein is reported to be 16 h (Shiraki and Guengerich, 1984). Therefore, the decline of apoprotein content and activities between 48 and 60 h may have been caused by loss of immunoreactivity or function via oxidative degradation of CYP1A1 by hyperoxia. Although cellular toxicity after prolonged hyperoxic exposure may explain in part the decrease in CYP1A1/1A2 expression at 60 h, the fact that the protein expression of other enzymes, such as glutathione *S*-transferase-α (Moorthy et al., 1997), and mRNA expression of CYC (Fig. 3A) were not attenuated at 60 h of hyperoxia suggests that the decline of induction by hyperoxia was relatively specific for CYP1A1, which may be of mechanistic relevance to hyperoxic lung injury. In fact, Paller and Jacob (1994) have provided evidence for P450 enzymes, upon degradation, as intracellular sources of redox-active iron, which might induce lung injury through increased formation of Fenton-like reactions or by propagating oxidative stress and lipid peroxidation (Kehrer and Smith, 1994; Yang et al., 1999; Moorthy, 2000).

Our data showing increases in CYP1A1/1A2 mRNA levels in liver at 48 h of hyperoxia (Fig. 4, A and B), followed by decline of CYP1A1/1A2 mRNA at 60 h, suggest that these phenomena are mediated by alteration of expression of the CYP1A1/1A2 genes. In contrast to the effects of hyperoxia on lung, wherein augmentation of CYP1A1 mRNA expression was observed after 24 h (Fig. 3), hepatic CYP1A1/1A2 expression was elevated after 48 h of hyperoxia (Fig. 4, A and B), suggesting tissue-specific differences in the regulation of CYP1A enzymes by hyperoxia.

The mechanisms of induction of CYP1A1/1A2 by hyperoxia in liver are not clearly understood. Our EMSA experiments (Fig. 5) strongly suggest interaction of an endogenous ligand with specific transcription factors (i.e., AHR-ARNT complex), which, upon binding to the AHREs of the CYP1A1 promoter, may modulate CYP1A1 gene expression (Okey et al., 1994). The protein-DNA complexes in the hyperoxic animals were of lower electrophoretic mobility than that observed in MC-treated animals, suggesting that an oxygen-sensitive coactivator, in addition to the AHR-ARNT complex, contributed to the regulation of CYP1A expression by hyperoxia. The presence of a supershifted band, albeit faint, in the presence of AHR antibodies supported the hypothesis that AHR-dependent mechanisms contributed to the modulation of CYP1A1 by hyperoxia. The presence of the putative oxygen-sensitive cofactor may have partially masked the AHR antibody reactive sites, which could explain the relatively weak super-

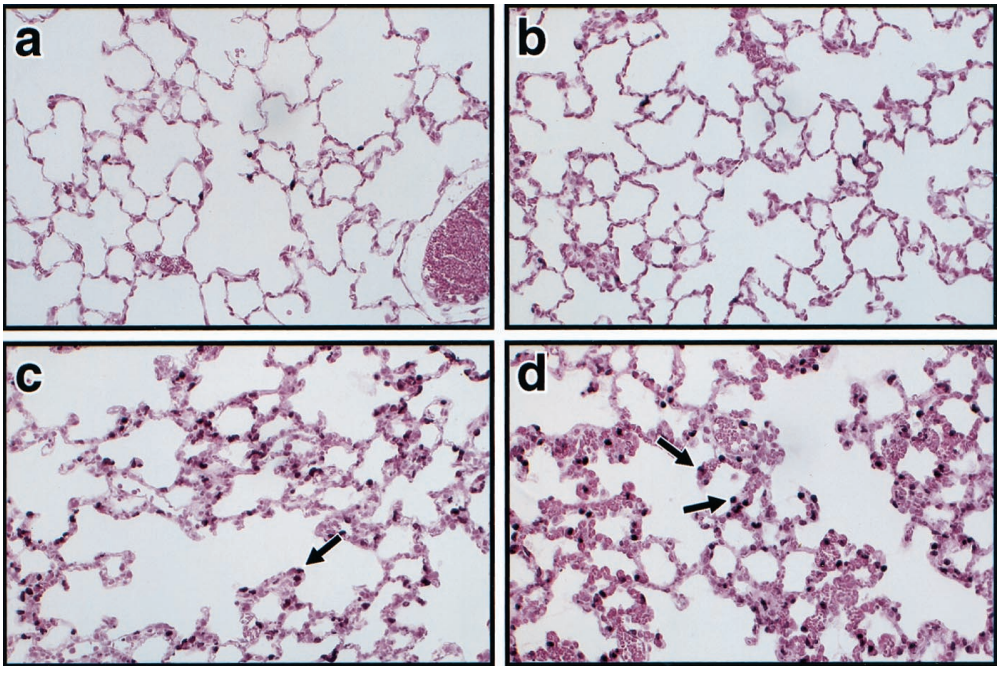

Fig. 8. Representative lung sections for MPO immunohistochemistry from rats maintained in room air (a) or exposed to hyperoxia for 24 (b), 48 (c), or 60 (d) h (original magnification, 66×). Air-breathing animals (a) and animals exposed to hyperoxia for 24 h (b) do not display significant number of MPO positive neutrophils. On the other hand, animals exposed to 48 (c) or 60 (d) h of hyperoxia show many MPO-positive neutrophils (arrow).

shited signal. Our observations showing AHR (−/−) mice to be refractory to CYP1A1 induction by hyperoxia (Fig. 9) strongly support the hypothesis that AHR-dependent mechanisms contribute to induction of CYP1A by hyperoxia.

The differential increases in the expression of immunoreactive CYP1A1, observed in the airway epithelial cells, type II pneumocytes, and endothelial cells between 24 and 48 h of hyperoxia (Figs. 6 and 7), indicates that induction of CYP1A1 enzyme in lung by hyperoxia occurs in a cell-specific manner. The major cell types in the airways are ciliated columnar cells and nonciliated cells termed Clara cells, which secrete CCSP. The observation that CYP1A1 (Fig. 6, a–d) colocalized with CCSP (Fig. 6e) indicated that CYP1A1 was expressed in Clara cells. Clara cells and type II pneumocytes of control rat lungs have been shown to contain CYP1A1, and treatment of rats with prototype CYP1A1 inducers such as MC leads to induction of CYP1A1 protein and mRNA in Clara cells (Parion et al., 1994). Although induction of CYP1A1 by MC also occurs in the type II pneumocytes, albeit to a lesser extent than in Clara cells, venous endothelial cells showed slight increase in CYP1A1 only after repeated administration of MC (Parion et al., 1994). Our findings showing enhanced immunoreactivity of CYP1A1 in the endothelial cells by hyperoxia are intriguing. Hazinski et al. (1995) reported that hyperoxia induces CYP1A1 in cultured endothelial cells, a phenomenon that is associated with microvascular injury in vivo. Because the endothelial cells are more vulnerable to injury, induction of CYP1A1 in the lung endothelium may have relevance to lung injury.

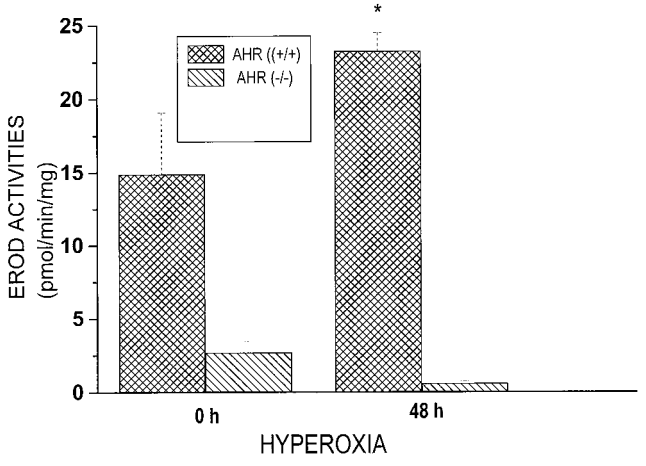

Fig. 9. EROD activities in lungs of AHR (+/+) and AHR (−/−) mice exposed to hyperoxia. AHR (+/+) or AHR (−/−) mice were exposed to room air or hyperoxia for 48 h, and EROD activities were measured in lung microsomes of these samples. Values represent means ± S.E. of data from at least 3 individual animals. [a], Different from air-breathing controls at $P < 0.05$.

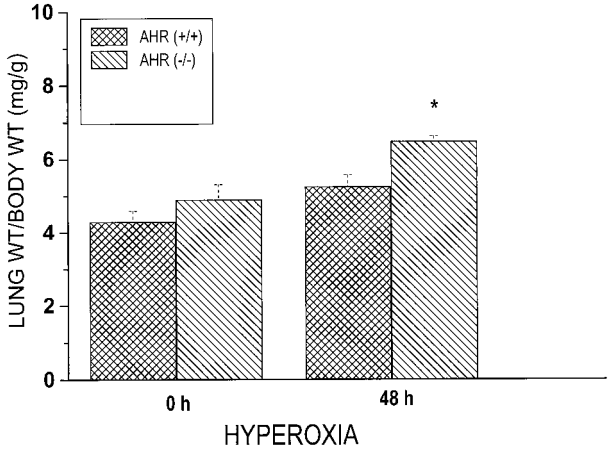

Fig. 10. Lung weight/body weight ratios of AHR (+/+) and AHR (−/−) animals exposed to hyperoxia. AHR (+/+) and AHR (−/−) animals were exposed to hyperoxia for 48 h, and ratios of right lung weight/body weight were determined as a measure of lung injury. [a], Significantly different from (+/+) animals at $P < 0.05$.

The increased neutrophil production in animals exposed to hyperoxia for 48 and 60 h, as determined by MPO-positive staining of lung sections (Fig. 8) indicated augmented inflammation of the lung by hyperoxia (Hudak et al., 1993; Parion et al., 1994). The following observations support the idea that modulation of CYP1A by hyperoxia has implications for hyperoxic lung injury. 1) Induction of pulmonary CYP1A1 mRNA, apoprotein, and activity after 24 to 48 h of hyperoxia, followed by dramatic decline of induction of pulmonary CYP1A1 parameters between 48 and 60 h (Figs. 1–3, 6, and 7), precedes massive lung inflammation (Fig. 8) and respiratory distress. 2) Pretreatment of rats with the ABT, which significantly inhibits CYP1A enzymes, followed by exposure of these animals to hyperoxia, severely potentiates lung injury (Moorthy, 2000). 3) Mice deficient in the gene for the AHR, which regulates the expression of CYP1A, are refractory to CYP1A1 induction (Fig. 9) and are more susceptible to hyperoxic lung injury than AHR (+/+) mice (Fig. 10). 4) The liver-specific CYP1A2 (−/−) mice are more sensitive to lung injury than CYP1A2 (+/+) mice (Moorthy et al., 1999).

In conclusion, based on the results obtained in the current study and information available in the literature, we propose a mechanistic hypothesis that would account for the effect of hyperoxia on pulmonary and hepatic CYP1A enzymes in relation to hyperoxic injury. The initial induction of CYP1A by hyperoxia (24–48 h) (Figs. 1–4, 6, and 7) may result in increased production of H_2O_2 (oxidative stress) (Morel et al., 1999, 2000), which in turn could attenuate CYP1A expression (Figs. 1–4 and 6) via mechanisms involving NF1 and basal transcription element on the CYP1A promoter (Morel et al., 2000) or through direct oxidative degradation of CYP1A enzymes. The degradation of CYP1A would lead to release of iron from the heme moiety, thereby contributing to the development of lung injury via Fenton-mediated free radical reactions such as lipid peroxidation (Kehrer and Smith, 1994; Yang et al., 1999; Moorthy, 2000). Additionally, oxidative stress, induced as a result of increased intracellular H_2O_2 or other ROS, could contribute to lung injury (Kehrer and Smith, 1994). CYP1A enzymes may also play beneficial role during hyperoxic exposures, as evidenced by increased susceptibility of CYP1A2 (−/−) (Moorthy et al., 1999) or AHR (−/−) mice (Fig. 10) to lung injury. Future studies directed toward understanding molecular mechanisms of P450 regulation by hyperoxia could lead to the development of specific strategies for the prevention/treatment of lung disease in infants and adults undergoing supplemental oxygen therapy.

Acknowledgments

We are grateful to Dr. Henry Strobel (Department of Biochemistry, University of Texas-Houston Medical School, Houston, TX) for helpful discussions and for critical review of the manuscript. We thank Dr. Paul Thomas (Rutgers University, Piscataway, NJ) for providing us antibody against CYP1A1 and Dr. Francesco Demayo

(Department of Cell Biology, Baylor College of Medicine, Houston, TX) for providing the CCSP antibody.

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