Disruption of the Ah Receptor Gene Alters the Susceptibility of Mice to Oxygen-Mediated Regulation of Pulmonary and Hepatic Cytochromes P4501A Expression and Exacerbates Hyperoxic Lung Injury

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ABSTRACT

Administration of supplemental oxygen is frequently encountered in infants suffering from pulmonary insufficiency and in adults with acute respiratory distress syndrome. However, hyperoxia causes acute lung damage in experimental animals. In the present study, we investigated the roles of the Ah receptor (AHR) in the modulation of cytochrome P4501A (CYP1A) enzymes and in the development of lung injury by hyperoxia. Adult male wild-type [AHR (+/+) mice and AHR-deficient animals [AHR (−/−)] were maintained in room air or exposed to hyperoxia (>95% oxygen) for 24 to 72 h, and pulmonary and hepatic expression of CYP1A and lung injury were studied. Hyperoxia caused significant increases in pulmonary and hepatic CYP1A1 activities (ethoxyresorufin O-deethylase) and mRNA levels in wild-type (C57BL/6J) AHR (+/+), but not AHR (−/−) mice, suggesting that AHR-dependent mechanisms contributed to CYP1A1 induction. On the other hand, hyperoxia augmented hepatic CYP1A2 expression in both wild-type and AHR (−/−) animals, suggesting that AHR-independent mechanisms contributed to the CYP1A2 regulation by hyperoxia. AHR (−/−) mice exposed to hyperoxia were more susceptible than wild-type mice to lung injury and inflammation, as indicated by significantly higher lung weight/body weight ratios, increased pulmonary edema, and enhanced neutrophil recruitment into the lungs. In conclusion, our results support the hypothesis that the hyperoxia induces CYP1A1, but not CYP1A2, expression in vivo by AHR-dependent mechanisms, a phenomenon that may mechanistically contribute to the beneficial effects of the AHR in hyperoxic lung injury.

Supplemental oxygen therapy is routinely needed in the treatment of pulmonary insufficiency, which is frequently encountered in preterm infants with respiratory distress and in older patients with acute respiratory distress syndrome (Fisher, 1980). However, hyperoxic therapy may contribute to tissue damage and the development of lung diseases, such as bronchopulmonary dysplasia in preterm infants (Smith and Welty, 1999). Exposure of experimental animals to hyperoxia causes lung injury, and although the underlying mechanisms are not completely understood, reactive oxygen species (ROS) (e.g., superoxide anion, hydrogen peroxide, and hydroxyl radical) are most likely candidates (Clark and Lambertsen, 1971; Smith and Welty, 1999).

Cytochrome P450 (P450) enzymes belong to a superfamily of heme proteins that play important roles in the metabolism of exogenous and endogenous chemicals (Guengerich, 1990). P450 enzymes, including CYP1A1, have also been implicated in the formation and further reactions of ROS and may also...

ABBREVIATIONS: ROS, reactive oxygen species; P450, cytochrome P450; AHR, Ah receptor; PAH, polycyclic aromatic hydrocarbon; MC, 3-methylcholanthrene; ABT, 1-aminobenzotriazole; EROD, ethoxyresorufin O-deethylase; MROD, methoxyresorufin O-demethylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; AHREs, Ah response elements; RT-PCR, reverse transcriptase-polymerase chain reaction; NF-2, nuclear transcription factor-E2; IL-1β, interleukin 1β.
play a role in pulmonary oxygen toxicity (Gonder et al., 1985; Mansour et al., 1988a,b; Hazinski et al., 1989, 1995; Okamoto et al., 1993; Gram, 1997; Morel et al., 1999; Couroucli et al., 2002).

The Ah receptor (AHR) is a cytosolic protein through which polycyclic aromatic hydrocarbons (PAHs) [e.g., 3-methylcholanthrene (MC)] induce a battery of enzymes that are encoded by the Ah gene locus (Nebert et al., 2000). The enzymes encoded by the genes of the Ah battery include CYP1A1, CYP1A2, glutathione S-transferase-α, NAD(P)H quinone reductase 1, UDP glucuronosyl transferase, and aldehyde dehydrogenase (Nebert et al., 2000). Induction of CYP1A1 by PAHs has been extensively studied (Denison and Phelan, 1998; Nebert et al., 2000). After entry into cells, the PAHs act as ligands for the AHR and bind to the AHR. The PAH-AHR complex enters the nucleus and binds to yet another protein termed AHR nuclear translocator. This ternary complex binds to AHR-Es, located in multiple copies on the CYP1A1 gene promoter (Denison and Phelan, 1998), leading to enhanced transcription of the CYP1A1 gene. In addition to the AHR-Es, basal transcriptional element on the proximal promoter also contributes to CYP1A1 induction via interaction with nuclear factor 1 (Morel et al., 1999). The mechanisms by which hyperoxia induces CYP1A1/1A2 in rodents is not completely understood, although AHR-dependent mechanisms have been suggested (Okamoto et al., 1993; Sindhu et al., 1999; Couroucli et al., 2002).

The possible involvement of AHR in hyperoxic lung injury was proposed earlier by Gonder et al. (1985), who showed that genetically inbred mouse strains that were responsive to induction of aryl hydrocarbon hydroxylase by PAHs (Ah-responsive) were more susceptible to hyperoxic lung injury than were Ah-nonresponsible strains. However, other correlative studies in strain-specific susceptibilities to hyperoxia do not clearly find strong associations between Ah responsiveness of animals and their sensitivities to hyperoxic injury, thereby suggesting that other factors (e.g., genetic background, diet, and antioxidant enzymes) need to be considered (Hudak et al., 1993).

When P450 activities are inhibited in rats with interferon inducers (Kikkawa et al., 1984) or lambs with cimetidine (Hazinski et al., 1989, 1995), hyperoxic lung injury is attenuated in these animals. However, the P450 inhibitor 1-aminobenzotriazole (ABT) severely potentiates lung damage by hyperoxia in rats (Moorthy et al., 2000). Species differences and/or specificities of inhibitors toward different P450 enzymes may explain the observed discrepancies. Although ABT inhibits CYP1A1/1A2 and CYP2B1/2B2 activities (Moorthy et al., 2000), cimetidine inactivates CYP2A6 and CYP2C11 but not CYP1A1/1A2, CYP2B1, or CYP3A1/1A2 (Levine et al., 1998). The unknown nonspecific effects of the pharmacological agents cannot be precluded. Pretreatment of rats (Mansour et al., 1988a) or mice (Mansour et al., 1988b) with CYP1A inducers protect animals from hyperoxic lung injury. These observations, in conjunction with our ABT studies (Moorthy et al., 2000), led to the hypothesis that CYP1A enzymes play a beneficial role in oxygen-mediated injury.

The existence of a mechanistic link between CYP1A expression and development of hyperoxic lung injury is further substantiated by our observations that hyperoxia elicits temporal modulation of hepatic and pulmonary CYP1A enzymes in rats (Couroucli et al., 2002), with CYP1A being induced after 24 to 48 h of hyperoxia, followed by decline at 60 h, the time point that correlates with onset of severe respiratory distress and lung injury. The mechanisms of CYP1A induction by hyperoxia and its role in hyperoxic lung injury are not completely understood. We therefore tested the hypotheses that 1) AHR-dependent mechanisms contribute to induction of pulmonary and hepatic CYP1A enzymes by hyperoxia, and 2) AHR (−/−) mice would be more susceptible to hyperoxic lung injury and inflammation than similarly exposed wild-type [AHR (+/+) ] mice.

Materials and Methods

Animals. We obtained breeding pairs of AHR (+/+) mice from Jackson Laboratories (Bar Harbor, ME). The AHR (−/−) mouse was originally developed in the laboratory of Chris Bradfield (University of Wisconsin, Madison, WI). These heterozygotes were bred in our facility. Tail DNA samples of the mice generated in our colony were genotyped by PCR and (+/+), (+/−), and (−/−) mice were identified (Schmidt et al., 1993). All mice were on pure C57BL/6J background.

Hyperoxia Exposure. Two-month-old male mice were used in the study. The animals were either maintained in room air or exposed to >95% O2 for 24, 48, or 72 h using pure O2 at 5 l/min, as we have described previously (Couroucli et al., 2002). Purified tap water and food (Purina Rodent Lab Chow 5001 from Purina Mills, Inc., Richmond, IN) were available ad libitum.

Perfusion and Tissue Harvesting. At the termination of their respective exposures, eight mice from each group were anesthetized with sodium pentobarbital (200 mg/kg) i.p. and euthanized by exsanguination while under deep pentobarbital anesthesia. In four mice 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 obtained for CYP1A1/1A2 analyses. 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 H2O) with zinc formalin after which the lungs were embedded in paraffin for subsequent histological and immunohistochemical analyses for assessing lung injury and neutrophil recruitment, respectively (Couroucli et al., 2002). The right lungs were used for subsequent RNA isolation and analyses.

Preparation of Microsomes and Enzyme Assays. Lung and liver microsomes were isolated by the calcium chloride precipitation method (Schmidt et al., 1993). All mice were on pure C57BL/6J background. The AHR (−/−) mouse was obtained from Jackson Laboratories (Bar Harbor, ME). The AHR (−/−) mouse was originally developed in the laboratory of Chris Bradfield (University of Wisconsin, Madison, WI). These heterozygotes were bred in our facility. Tail DNA samples of the mice generated in our colony were genotyped by PCR and (+/+), (+/−), and (−/−) mice were identified (Schmidt et al., 1993). All mice were on pure C57BL/6J background.

The primary monoclonal antibody to CYP1A1, which cross-reacts with CYP1A2, was a generous gift from Dr. P. E. Thomas (Rutgers University, Piscataway, NJ). Goat anti-mouse IgG conjugated with horseradish peroxidase was from Bio-Rad (Hercules, CA). The primary monoclonal antibody to CYP1A1, which cross-reacts with CYP1A2, was a generous gift from Dr. Frank Gonzalez of the National Cancer Institute, Bethesda, MD. Anti-neutrophil antibody was from Bio-Rad. All real-time reverse transcriptase-polymerase chain reaction (RT-PCR) reagents were from Applied Biosystems (Foster City, 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 from individual animals, as reported previously (Couroucli et al., 2002). Liver microsomes were isolated by the calcium chloride precipitation method (Cinti et al., 1972; 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 ac-
cording to the method of Pohl and Fouts (1980), 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 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). Quantitation of the blots was accomplished by laser densitometric scanning of the blots, as described previously (Moorthy et al., 2000).

**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/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 32P-labeled CYP1A1 cDNA probe (20 × 106 cpm) (Moorthy, 2000; Couroucli et al., 2002). GAPDH cDNA probe was used as an internal control to assess RNA transfer, loading, and hybridization.

**Real-Time RT-PCR Assays.** Total RNA (50 ng) from lungs or livers of air-breathing and hyperoxic animals was subjected to one step real-time quantitative TaqMan RT-PCR. ABI PRISM 7700 Sequence Detection System (Applied Biosystems) was used for the RT-PCR reactions. 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. Following a RT hold for 30 min at 48°C, the samples were denatured at 95°C for 10 min. The thermal cycling step was for 40 cycles 95°C, 1 min; 55°C, 1 min; and 72°C, 1 min. Serial dilutions of RNA were used to optimize and validate RT-PCR conditions for CYP1A1, CYP1A2, and 18S genes. Each data point was repeated three times. Quantitative values were obtained from the threshold PCR cycle number (Ct) at which the increase in signal was associated with an exponential growth for PCR product starts to be detected. The relative mRNA levels for CYP1A1 or 1A2 were normalized to their 18S content. The relative expression levels of the target gene were calculated according to the equation, 2ΔΔCt, where ΔΔCt = Ct_target gene - Ct_18S gene.

**Lung Weight/Body Weight Ratios.** Lung weight/body weight ratios were calculated as an index of lung injury in animals whose lungs were not perfused for isolation of microsomes.

**Lung Histology and Immunohistochemistry.** Routine histology was performed on lung tissues from individual animals following staining of the paraffin sections with hematoxylin and eosin (Ramsay et al., 1998). Neutrophil recruitment into lungs was assessed by immunohistochemistry, in the presence of anti-neutrophil antibodies (Ramsey et al., 1998).

**Statistical Analyses.** Data are expressed as means ± S.E. Student’s t test and one-way analysis of variance (ANOVA), followed by post hoc Newman Keuls, were used to assess significant differences arising from exposure to hyperoxia in the AHR (+/+) and AHR (−/−) animals for different time points. Two-way ANOVA was also performed using exposure to hyperoxia and genotype as variables to determine the statistical significance in the effect of each of the variables and a possible interaction between them. P values <0.05 were considered significant.

**Results**

In the present study, we tested the central hypothesis that the AHR plays a role in the induction of CYP1A1 by hyperoxia and that mice lacking the gene for the AHR would be more susceptible to hyperoxic lung injury than wild-type mice.

The AHR Contributes to Induction of Pulmonary CYP1A1 by Hyperoxia. AHR (+/+) mice exposed to hyperoxia for 48 h displayed a 4-fold statistically significant increase in pulmonary EROD (CYP1A1) activities over those of air-breathing animals (Fig. 1). However, AHR (−/−) mice were refractory to induction of CYP1A1 by hyperoxia (Fig. 1). Two-way ANOVA indicated a significant effect of genotype and exposure to hyperoxia and a significant interaction between the two variables. The basal pulmonary EROD activities in air-breathing AHR (−/−) animals were much lower than those of wild-type animals (Fig. 1), indicating that endogenous CYP1A1 expression is dependent upon the presence of the AHR. Real-time RT-PCR analyses (Fig. 2) indicated that hyperoxia-induced pulmonary CYP1A1 mRNA expression in the wild-type, but not AHR-null animals, suggested that AHR-dependent mechanisms play an important role in the induction of CYP1A1 by hyperoxia. Quantitative analyses indicated that CYP1A1 mRNA of AHR (+/+) animals was induced by 3- and 13-fold after 24 and 48 h of hyperoxia, respectively, but this induction declined after 72 h (Fig. 2). In the AHR-null animals, the basal expression of CYP1A1 mRNA was significantly lower than that observed in wild-type animals (Fig. 2). Hyperoxia appeared to elicit a modest increase in the expression of CYP1A1 after 48 h of hyperoxia, but this was not statistically significant. By 72 h, CYP1A1 levels were lesser than those of air-breathing animals (Fig. 2).

**Effects of Hyperoxia on Hepatic EROD and MROD Activities.** To determine whether the induction of the liver CYP1A1 enzyme by hyperoxia is mediated by the AHR, we exposed AHR (+/+) or AHR (−/−) mice to hyperoxia for 24, 48, or 72 h and measured the activities of EROD and MROD in liver microsomes. In AHR (+/+) animals, hyperoxia elicited a 2-fold induction of hepatic EROD activities after 24 h (Table 1). After 48 h, hepatic EROD activities were increased by 3.5-fold. At the 72-h time point, the EROD activities were significantly lower than air-breathing animals (Table 1). In the AHR (−/−) animals, no induction of CYP1A1 activities was observed at any of the time points. Two-way ANOVA indicated a significant effect of genotype and exposure to hyperoxia and a significant interaction between the two variables. Hepatic MROD activities were increased in the wild-type animals by 210 and 300% after hyperoxia for 24 and
and Methods

Values represent means ± S.E. of data from at least three individual animals. a, different from air-breathing controls at P < 0.05, b, different from wild-type air-breathers at P < 0.05.

TABLE 1

Effect of hyperoxia on hepatic EROD and MROD activities

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hyperoxia</th>
<th>EROD (pmol/min/mg protein)</th>
<th>MROD (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR (+/+)</td>
<td>0</td>
<td>491 ± 51</td>
<td>908 ± 87</td>
</tr>
<tr>
<td>AHR (+/+)</td>
<td>24</td>
<td>980 ± 100</td>
<td>1920 ± 145</td>
</tr>
<tr>
<td>AHR (+/+)</td>
<td>48</td>
<td>794 ± 87</td>
<td>2785 ± 380</td>
</tr>
<tr>
<td>AHR (+/+)</td>
<td>72</td>
<td>209 ± 16</td>
<td>670 ± 54</td>
</tr>
<tr>
<td>AHR (+/-)</td>
<td>0</td>
<td>248 ± 45</td>
<td>304 ± 59</td>
</tr>
<tr>
<td>AHR (+/-)</td>
<td>24</td>
<td>335 ± 64</td>
<td>863 ± 92</td>
</tr>
<tr>
<td>AHR (+/-)</td>
<td>48</td>
<td>326 ± 49</td>
<td>1303 ± 170</td>
</tr>
<tr>
<td>AHR (+/-)</td>
<td>72</td>
<td>101 ± 17</td>
<td>280 ± 32</td>
</tr>
</tbody>
</table>

a Different from air-breathing controls at P < 0.05.
b Different from wild-type air-breathers at P < 0.05.

48 h, respectively, over the corresponding activities in the air-breathing animals. However, after 72 h of hyperoxia, the activities declined (Table 1). The constitutive expression of MROD activities was much lower in the AHR (−/−) animals, compared with wild-type animals. However, 24 h of hyperoxia caused a 3-fold induction of MROD activities in the AHR (−/−) animals (Table 1). Although 48 h of hyperoxia still showed elevated MROD activities, by 72 h, MROD activities were not different from room air controls (Table 1). Two-way ANOVA indicated a significant effect of genotype and exposure to hyperoxia and a significant interaction between the two variables.

Effects of Hyperoxia on Hepatic CYP1A1/1A2 Apoproteins. In an effort to determine whether modulation of CYP1A1/1A2 activities would be accompanied by changes in the corresponding apoproteins, Western blotting of liver microsomal samples isolated from AHR (+/+) and AHR (−/−) animals was performed. CYP1A1 apoprotein was not detectable in air-breathing or hyperoxic animals in our Western blots (Fig. 3). In contrast, CYP1A2 protein was observed in all samples. As shown in the figure, hyperoxia for 48 h significantly induced (1.8-fold) (quantitative data not shown) CYP1A2 apoproteins in the wild-type animals compared with air-breathing animals. In the AHR-null animals, the induction of CYP1A2 expression by hyperoxia for 48 h was more pronounced (3-fold increase) than that observed in similarly exposed wild-type mice (Fig. 3).

Effects of Hyperoxia on Hepatic CYP1A1/1A2 mRNA. To determine whether modulation of CYP1A1/1A2 by hyperoxia is preceded by similar alteration in the corresponding message levels, we performed Northern hybridization of total RNA from livers of AHR (+/+), AHR (−/−), and wild-type mice that were either maintained in room air or were exposed to hyperoxia for different lengths of time. CYP1A1 was not detectable under any conditions in either AHR (+/+) or AHR (−/−) animals (Fig. 4). Both CYP1A1 (23S) and CYP1A2 (18S) transcripts were detectable in livers of mice exposed to MC (Fig. 4). In AHR (+/+) animals, hyperoxia for 48 h elicited a 3.5-fold increase in CYP1A2 mRNA expression compared with that of air-breathing animals (Fig. 4, Table 2). By 72 h, the induction of CYP1A2 expression declined. In the AHR (−/−) mice, hyperoxia for 24 and 48 h resulted, respectively, in a 4.8- and 6.7-fold augmentation in the expression of CYP1A2 mRNA compared with that of air-breathing animals.
Table 2: Quantitation of hepatic CYP1A2 mRNA levels

The Northern blots, obtained from experiments described in the legend to Fig. 4, were subjected to phosphor imaging analyses, and the CYP1A2 mRNA levels were estimated in individual animals. Data represent means ± S.E. of data from at least three individual animals.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hyperoxia (h)</th>
<th>Pixel Density × 10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR (+/+)</td>
<td>0</td>
<td>21.0 ± 0.82</td>
</tr>
<tr>
<td>AHR (+/+)</td>
<td>24</td>
<td>25.0 ± 2.3</td>
</tr>
<tr>
<td>AHR (+/-)</td>
<td>48</td>
<td>72.0 ± 9.3a</td>
</tr>
<tr>
<td>AHR (+/-)</td>
<td>72</td>
<td>33.0 ± 4.2a</td>
</tr>
<tr>
<td>AHR (−/−)</td>
<td>0</td>
<td>7.7 ± 0.18b</td>
</tr>
<tr>
<td>AHR (−/−)</td>
<td>24</td>
<td>37.0 ± 4.2a</td>
</tr>
<tr>
<td>AHR (−/−)</td>
<td>48</td>
<td>52.0 ± 6.7a</td>
</tr>
<tr>
<td>AHR (−/−)</td>
<td>72</td>
<td>6.7 ± 3.2</td>
</tr>
</tbody>
</table>

a Different from air-breathing controls at P < 0.05.
b Different from wild-type air-breathers at P < 0.05.

After 72 h of hyperoxia, the levels of CYP1A2 mRNA were similar to those of animals maintained in room air (Table 2). Hyperoxia exposure did not elicit significant changes in the mRNA levels of the housekeeping transcript GAPDH (Fig. 4, quantitative data not shown).

Since hepatic CYP1A1 mRNA was not detectable by Northern hybridization, we performed real-time RT-PCR. As shown in Fig. 5, hyperoxia for 24 to 48 h augmented levels of CYP1A1 in the wild-type animals. The induction declined by 72 h (Fig. 5). In air-breathing AHR-null animals, the CYP1A1 mRNA expression was significantly lesser than air-breathing wild-type animals. Hyperoxia did not alter CYP1A1 mRNA expression in the AHR-null animals (Fig. 5).

We verified our Northern analyses on the CYP1A2 mRNA by performing quantitative real-time RT-PCR. In the AHR (+/+ ) animals, hyperoxia for 48 h resulted in a 2.6-fold increase in CYP1A2 message compared with that in animals that were maintained in room air (Fig. 6). The induction of CYP1A2 mRNA declined after 72 h of hyperoxia (Fig. 6). In the AHR (−/−) animals, hyperoxia for 24 to 48 h elicited a 3.5- to 6-fold increase in the levels of CYP1A2 mRNA compared with air-breathing animals (Fig. 6).

Fig. 5. RT-PCR analyses showing the effects of hyperoxia on hepatic CYP1A1 mRNA of AHR (+/+) and AHR (−/−) animals. AHR (+/+) or AHR (−/−) animals were maintained in room air or exposed to hyperoxia for 24 to 72 h, and hepatic CYP1A1 mRNA was analyzed by RT-PCR, as described under Materials and Methods. Data represent relative-fold changes caused by hyperoxia on CYP1A1 gene expression compared with that in AHR (+/+) animals. Values represent means ± S.E. of data from at least three individual animals. a, different from air-breathing controls at P < 0.05. b, different from wild-type air-breathers at P < 0.05.

Fig. 6. RT-PCR analyses showing the effects of hyperoxia on hepatic CYP1A2 mRNA of AHR (+/+) and AHR (−/−) animals. AHR (+/+) or AHR (−/−) animals were maintained in room air or exposed to hyperoxia for 24 to 72 h, and hepatic CYP1A2 mRNA was analyzed by RT-PCR, as described under Materials and Methods. Data represent relative-fold changes caused by hyperoxia on CYP1A2 gene expression compared with that in AHR (+/+) animals. Values represent means ± S.E. of data from at least three individual animals. a, different from air-breathing controls at P < 0.05. b, different from wild-type air-breathers at P < 0.05.

Lung Injury and Inflammation. With the exception of one AHR (−/−) animal, which died between 60 and 72 h of hyperoxia, all animals survived 72 h of hyperoxia. The AHR (−/−) mice exposed to 48 h of hyperoxia had significantly higher lung weight/body weight ratios than similarly exposed wild-type mice, suggesting that AHR (−/−) mice were more susceptible to hyperoxic lung injury than the AHR (+/+) mice (Fig. 7). Two-way ANOVA indicated a significant effect of exposure to hyperoxia, but not an effect of genotype, and a significant interaction between the two variables.

Histological analyses also revealed severe alveolar flooding and pulmonary edema in lungs of AHR (−/−) mice exposed to 72 h of hyperoxia compared with similarly exposed wild-type mice (Fig. 8). Lungs of air-breathing AHR (+/+), as well as AHR (−/−) animals, showed normal lung architecture and showed no evidence of lung injury (not shown). Immunohistochemistry using anti-neutrophil antibodies indicated greater recruitment...
of neutrophils into lungs of AHR-null versus wild-type animals (Fig. 9). Neutrophil infiltration was minimal in air-breathing AHR (+/H11001/H11001) as well as AHR (+/H11002/H11002) animals (not shown).

### Discussion

In this study, we investigated the role of the AHR in modulation of CYP1A enzymes by hyperoxia, in relation to lung injury. The marked increases (~4-fold) in lung EROD activities (Fig. 1) caused by exposure to hyperoxia for 48 h in the wild-type animals indicate induction of CYP1A1, as EROD activities are relatively specific for CYP1A1 (Moorthy, 2000; Couroucli et al., 2002). The results are in agreement with those obtained in male Sprague-Dawley rats (Moorthy et al., 1997), wherein induction of pulmonary CYP1A1 activities was observed after 48 h of hyperoxia followed by decline between 48 and 72 h of hyperoxia in the wild-type mice (Fig. 1), a phenomenon that coincides with development of severe respiratory distress.

The increase in lung CYP1A1 mRNA expression after 24 to 72 h of hyperoxia in the wild-type animals (Fig. 2) supported the hypothesis that induction of CYP1A1 activities was due, in part, to activation of CYP1A1 gene expression. Hazinski et al. (1995) have shown induction of CYP1A1 mRNA by hyperoxia in cultured endothelial cells from lambs to be mediated by transcriptional mechanisms. The fact that the expression of CYP1A1 mRNA declined after 72 h of hyperoxia strongly suggests that the decline of EROD activities at this time point was due to down-regulation of CYP1A1 expression at the pretranslational level. Morel et al. (1999) have shown CYP1A1-dependent increases in the formation of H₂O₂ in
human hepatoma cells treated with benzo[a]pyrene. Because H$_2$O$_2$ is formed in response to hyperoxia (Freeman and Crapo, 1981), it is possible that the increases in H$_2$O$_2$ production may have attenuated CYP1A1 gene expression by an autoregulatory loop mechanism involving down-regulation of nuclear factor 1, a protein whose binding to this site on the basal transcription element of the CYP1A1 promoter is critical to the expression of the gene (Morel et al., 1999). The fact that hyperoxia did not significantly induce CYP1A1 activities or mRNA levels in the AHR (−/−) animals supports the hypothesis that hyperoxia induces CYP1A1 by AHR-dependent mechanisms.

The increases in liver EROD activities by hyperoxia in AHR (+/+)(Table 1), but not AHR (−/−) animals, supported the hypothesis that, similar to lung, induction of hepatic CYP1A1 by hyperoxia was also mediated by AHR-dependent mechanisms. Sindhu et al. have demonstrated that ozone-oxidized tryptophan, which is a ligand for the AHR, induces CYP1A1 (Sindhu et al., 1999) as well as CYP1A2 (Sindhu et al., 2000) in mouse hepatoma cells. Since hyperoxia could oxidize tryptophan or other aromatic amino acids that could act as ligands for the AHR, it is possible that hyperoxia induced CYP1A1 enzyme in vivo via such a mechanism.

The increases in hepatic EROD activities and MROD were paralleled by augmentation of CYP1A2 protein contents (Fig. 3) and CYP1A1/1A2 mRNA levels (Figs. 4–6), suggesting that hyperoxia induced CYP1A enzymes by transcriptional or post-transcriptional mechanisms. The absence of CYP1A1 apoprotein in livers of air-breathing or hyperoxic mice (Fig. 3) could be attributed to its levels being lower than the limits of detection on the Western blots.

In contrast to the induction of pulmonary and hepatic CYP1A1 enzymes that was primarily mediated by AHR-dependent mechanisms, the induction of CYP1A2 appeared to be induced by AHR-independent mechanisms, since hyperoxia significantly induced CYP1A2 expression in wild-type and AHR-null animals (Figs. 3, 4, and 6). There is precedence of induction of CYP1A2 by chemicals such as phenobarbital, which also elicit induction by AHR-independent mechanisms (Sakuma et al., 1999). In this regard, it appears that CYP1A1 and 1A2 are differentially regulated by hyperoxia. This is in contrast to the findings of Sindhu et al. (1999, 2000), who showed oxidized tryptophan to induce CYP1A1 as well as 1A2 in mouse hepatoma cells. The fact that CYP1A2 induction was more pronounced in the AHR (−/−) animals compared with wild-type animals may have been due to compensatory mechanisms resulting from depleted expression of CYP1A1 in these animals.

The decrease in the expression of CYP1A1/1A2 after 48 to 72 h of hyperoxia may have been most probably due to decrease in the transcription of the corresponding genes after prolonged hyperoxia (Hazinski et al., 1995; Couroucli et al., 2002). The attenuation in the expression of CYP1A1/1A2 apoprotein between 48 and 72 h of hyperoxia may also have involved other mechanisms. The half-life of CYP1A1/1A2 proteins is reported to be 16 h (Shiraki and Guengerich, 1984). Therefore, the decline of apoprotein content and activities between 48 and 72 h may have been due to loss of immuno-reactivity or function via oxidative degradation of CYP1A1/1A2 by hyperoxia. Although cellular toxicity after prolonged hyperoxic exposure may, in part, explain the decrease in CYP1A1/1A2 expression at 72 h, the fact that the protein expression of other enzymes such as glutathione S-transferase-α (Moorthy et al., 1997) and mRNA expression of cyclophilin (Couroucli et al., 2002) were not attenuated after prolonged hyperoxia suggests that the decline of induction by hyperoxia was relatively specific for CYP1A1/1A2, 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 (Paller and Jacob, 1994; Smith and Welty, 1999). Zhang et al. (2003) have recently shown that hyperoxia may exacerbate lung injury by increasing lung epithelial cell death. Since CYP1A enzymes are highly expressed in pulmonary epithelial cells (Couroucli et al., 2002), it is possible that decreased CYP1A expression in these cells after prolonged hyperoxia (60–72 h) may have contributed to oxygen-induced lung damage.

The significance of modulation of hepatic CYP1A enzymes by hyperoxia is not well understood. Our results showing increased susceptibility of the lung-specific cytochrome P450 enzyme CYP1A2-null animals to hyperoxic lung injury (Moorthy et al., 1999) suggests that hepatic CYP1A enzymes play a beneficial role in hyperoxic lung injury.

The increased pulmonary edema (Figs. 7 and 8) and neutrophil production in wild-type animals exposed to hyperoxia for 72 h, as determined by anti-neutrophil-positive staining of lung sections (Fig. 9), indicated augmented inflammation of the lung by hyperoxia (Couroucli et al., 2002). The observation that AHR (−/−) animals displayed greater extent of oxygen-mediated lung injury and inflammation than similarly exposed wild-type animals suggested that AHR plays a beneficial role in the protection against lung injury. Because the AHR regulates the induction of CYP1A enzymes, it is possible that suppression of the expression of these enzymes, which may play a role in the detoxication of ROS, may have contributed to the greater sensitivities of the AHR (−/−) animals to hyperoxic lung injury.

Although phase II or antioxidant enzymes (e.g., superoxide dismutase, catalase) may also have contributed to some of the beneficial effects of the AHR, which may up-regulate superoxide dismutase through the AHR (Park and Rho, 2002), the fact that these enzymes are not induced in wild-type adult mice exposed to hyperoxia (Frank, 1991) argue against a critical role for these enzymes in the beneficial effects of the AHR in hyperoxic lung injury. However, the recent finding that nuclear transcription factor-E2 (Nrf-2), which regulates many phase II genes, protects against hyperoxic lung injury (Cho et al., 2001) suggests that some of the phase II/antioxidant enzymes regulated by Nrf-2 may have played a protective role in wild-type mice exposed to hyperoxia. For example, over-expression of heme oxygenase-1, which is regulated by Nrf-2 in human pulmonary epithelial cells, results in cell growth arrest and increased resistance to hyperoxia (Lee et al., 1996).

Since the AHR also regulates proteins other than CYP1A and phase II/antioxidant enzymes, such as those involved in cell cycle regulation and apoptosis (Nebert et al., 2000), it is possible that these proteins may also have contributed to the beneficial effects against oxygen injury. For example, it has been reported that growth regulatory factors such as interleukin 1-β (IL-1β), which attenuates hyperoxic lung injury
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