Omeprazole Attenuates Hyperoxic Lung Injury in Mice via Aryl Hydrocarbon Receptor Activation and Is Associated with Increased Expression of Cytochrome P4501A Enzymes

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ABSTRACT

Hyperoxia contributes to lung injury in experimental animals and bronchopulmonary dysplasia (BPD) in preterm infants. Cytochrome P4501A (CYP1A) enzymes, which are regulated by the aryl hydrocarbon receptor (AhR), have been shown to attenuate hyperoxic lung injury in rodents. Omeprazole, a proton pump inhibitor, used in humans to treat gastric acid-related disorders, induces hepatic CYP1A *in vitro*. However, the mechanism by which omeprazole induces CYP1A and its impact on CYP1A expression *in vivo* and hyperoxic lung injury are unknown. Therefore, we tested the hypothesis that omeprazole attenuates hyperoxic lung injury in adult wild-type (WT) C57BL/6J mice by an AhR-mediated induction of pulmonary and hepatic CYP1A enzymes. Accordingly, we determined the effects of omeprazole on pulmonary and hepatic CYP1A ex-

pression and hyperoxic lung injury in adult WT and AhR dysfunctional (AhRd) mice. We found that omeprazole attenuated lung injury in WT mice. Attenuation of lung injury by omeprazole paralleled enhanced pulmonary CYP1A1 and hepatic CYP1A2 expression in the omeprazole-treated mice. On the other hand, omeprazole failed to enhance pulmonary CYP1A1 and hepatic CYP1A2 expression and protect against hyperoxic lung injury in AhRd mice. In conclusion, our results suggest that omeprazole attenuates hyperoxic lung injury in mice by AhR-mediated mechanisms, and this phenomenon is associated with induction of CYP1A enzymes. These studies have important implications for the prevention and/or treatment of hyperoxia-induced disorders such as BPD in infants and acute respiratory distress syndrome in older children and adults.

Introduction

Bronchopulmonary dysplasia (BPD) is the most common and extensively studied complication of prematurity (Baraldi and Filippone, 2007). Affected infants are more likely to have long-term pulmonary problems, increased rehospitalizations during the first year of life, and delayed neurodevelopment (Short et al., 2003; Fanaroff et al., 2007). The etiology of BPD is probably multifactorial, and hyperoxia-induced generation of reactive oxygen species is thought to contribute to lung injury via oxidation of biologically important cellular macromolecules (Freeman and Crapo, 1981). It is also known that

exposure of experimental animals such as rodents to hyperoxia causes lung damage (Clark and Lambertsen, 1971), which makes them an ideal model to investigate the mechanisms and rational therapeutic interventions for BPD.

The cytochrome P450 enzymes belong to a superfamily of hemoproteins that are involved in the metabolism of exogenous and endogenous chemicals (Guengerich, 1990). The cytochrome P4501A (CYP1A) enzymes are of particular interest to oxygen toxicity. The CYP1A subfamily has two isoforms, CYP1A1 and CYP1A2. CYP1A1 is essentially an extrahepatic enzyme that is predominantly present in rodent and human lungs, intestines, placenta, and kidneys. CYP1A2 is expressed mainly in the rodent and human liver and is not or is weakly expressed in extrahepatic tissues. Hyperoxia for 48 h induces CYP1A1/CYP1A2 in liver and CYP1A1 in lung of adult rats. It is noteworthy that the induction of CYP1A enzymes in liver and lung declines after continuation of hyperoxia for 60 h (Moorthy et al., 1997; Couroucli et al., 2002), the time period that coincides with expression of overt respi-

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ABBREVIATIONS: BPD, bronchopulmonary dysplasia; AhR, aryl hydrocarbon receptor; AhRd, AhR dysfunctional; BNF, β -naphthoflavone; CYP1A, cytochrome P4501A; EROD, ethoxyresorufin O-deethylase; 3-MC, 3-methylcholanthrene; MROD, methoxyresorufin O-demethylase; MCP-1, monocyte chemoattractant protein-1; WT, wild type; RT-PCR, reverse transcriptase-polymerase chain reaction; LW, lung weight; BW, body weight.

ratory distress in these animals, suggesting that CYP1A induction may protect against hyperoxic lung injury. The protection against hyperoxic lung injury of adult rodents pretreated with β -naphthoflavone (BNF) (Sinha et al., 2005) or 3-methylcholanthrene (3-MC) (Mansour et al., 1988) has been attributed to the aryl hydrocarbon receptor (AhR)-mediated induction of CYP1A1, an enzyme with high peroxidase activity. It has also been shown that the CYP1A inhibitor 1-aminobenzotriazole potentiates hyperoxic lung injury in rats (Moorthy et al., 2000). Although direct evidence is lacking, CYP1A1 has been implicated in the metabolism of F₂-isoprostanes (Tong et al., 2003).

The tissue-specific up-regulation of the CYP1A enzymes by classic inducers such as 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin, 3-MC, and BNF occurs via the AhR-dependent mechanism (Sinal et al., 1999; Nebert et al., 2004). The classic inducers serve as ligands and bind to the AhR after entry into the cells. This results in translocation of AhR into the nucleus. In the nucleus, the AhR heterodimerizes with the AhR nuclear translocator, and the heterodimer interacts with Ah-responsive elements, located as multiple copies within CYP1A1 gene promoter, leading to enhanced transcription of the CYP1A1 gene.

Omeprazole, a substituted benzimidazole derivative, is a proton pump inhibitor that inhibits gastric acid secretion in both humans (Lind et al., 1983) and animals (Larsson et al., 1983). Omeprazole is 97% bound to plasma proteins and is extensively metabolized in the liver via CYP2C19 and CYP3A4 to hydroxylated sulfinyl and sulfone derivatives, with little unchanged drug excreted in the urine. It has been widely used in the management of gastric acid-related disorders in humans for approximately 15 years (Li et al., 2004). Previous studies have shown that omeprazole can induce CYP1A1/2 at mRNA, protein, and enzyme levels in human and animal hepatocytes in vitro (Diaz et al., 1990; Krusekopf et al., 1997). However, the impact of omeprazole on pulmonary and hepatic CYP1A enzymes in vivo and its role in hyperoxic lung injury in mice are unknown. Likewise, there is little information available on the precise role of AhR in the induction of CYP1A by omeprazole. In the current study, we tested the hypothesis that omeprazole will induce pulmonary and hepatic CYP1A enzymes by AhR-dependent mechanisms and attenuate hyperoxic lung injury in mice. We hereby demonstrate a novel protective role of omeprazole against hyperoxic lung injury in mice by an AhR-mediated mechanism, and this phenomenon is associated with induction of CYP1A enzymes. Our findings indicate a potential role of omeprazole in the prevention and/or treatment of hyperoxia-induced lung disorders such as BPD in human preterm infants and acute respiratory distress syndrome in older children and adults.

Materials and Methods

Animals. This study was conducted in accordance with the federal guidelines for the humane care and use of laboratory animals and was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. The aryl hydrocarbon receptor dysfunctional (AhRd) B6.D2N-Ahr^d/J strain mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Dr. Daniel Nebert (University of Cincinnati, Cincinnati, OH) initially backcrossed Ahr^d allele from DBA/2N onto C57BL/6N via a backcross-intercross breeding scheme and transferred this congenic to Dr. Alan Poland (Uni-

versity of Wisconsin, Madison, WI) at generation N13, who then backcrossed the Ahr^d allele onto C57BL/6J, again via a backcross-intercross breeding scheme. The resulting homozygotes at or beyond generation N17 were maintained at The Jackson Laboratory by sibling intercross. Eight-week-old male C57BL/6J wild-type (WT) and AhRd mice maintained at the Texas Children's Hospital animal facility were used for the study. They were fed standard mice food and water ad libitum. Animals were maintained in 12-h day/night cycles.

Chemicals. Omeprazole, calcium chloride, Tris, sucrose, NADPH, bovine serum albumin, ethoxyresorufin, glutathione reductase, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich (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, was a generous gift from Dr. Paul E. Thomas (Rutgers University, Piscataway, NJ). Goat anti-mouse IgG conjugated with horseradish peroxidase and anti-neutrophil antibody were from Bio-Rad Laboratories. All real-time reverse transcriptase-polymerase chain reaction (RT-PCR) reagents were from Applied Biosystems (Foster City, CA).

Experiment Design. In brief, we used a total of 32 WT mice and 32 AhRD mice in the study. The mice were injected intraperitoneally with either 50 mg/kg/day of omeprazole (dissolved in 100 μ l of corn oil; n = 16/genotype) or 100 μ l of corn oil (controls; n = 16/genotype) once daily for 5 days. The mice were then maintained in either room air (21% oxygen) or exposed to a hyperoxic (95–100% oxygen) environment using pure O₂ at 5 l/min for 72 h in a sealed Plexiglas chamber as reported previously (Gonder et al., 1985). After sealing, the oxygen concentration in the Plexiglas chamber was checked frequently by an analyzer (Ventronics, Kenilworth, NJ). Purified tap water and food (Purina Rodent Lab Chow 5001; Purina Mills, Inc., Richmond, IN) were available ad libitum. After 72 h of hyperoxia exposure, the animals were anesthetized with sodium pentobarbital (200 mg/kg i.p.) and euthanized by exsanguination while under deep pentobarbital anesthesia. The lung and liver tissues were harvested for analysis of CYP1A induction and hyperoxic lung injury. Our preliminary dose-responsive studies in wild-type mice after administration of 10 to 150 mg/kg/day i.p. of omeprazole for 3 days showed that only doses more than 50 mg/kg/day increased CYP1A enzyme activities (data not shown). Therefore, we selected an omeprazole dose of 50 mg/kg/day for our studies in mice.

Preparation of Microsomes and Enzyme Assays. Lung and liver samples at the time of dissection were frozen immediately with liquid nitrogen and maintained at a temperature of -80°C until preparation of microsomes. 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 (Moorthy et al., 1997). Ethoxyresorufin *O*-deethylase (EROD) (CYP1A1) activities in lung and liver microsomes and methoxyresorufin *O*-demethylase (MROD) (CYP1A2) activities in liver microsomes were assayed as described previously (Moorthy et al., 1997).

Western Blotting. Lung and liver microsomes (5 μ g of protein) prepared from individual animals was subjected to SDS-polyacrylamide gel electrophoresis in 7.5% acrylamide gels. The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by Western blotting. For the Western blot analysis, a monoclonal antibody to CYP1A1, which cross-reacts with CYP1A2, was used as a primary antibody. The primary antibody was detected by incubation with the horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody. The immuno-reactive bands were detected by chemiluminescence methods, and the band density was analyzed by Kodak 1D 3.6 imaging software (Eastman Kodak, Rochester, NY).

Real-Time RT-PCR Assays. Total mRNA was isolated using a modification of the procedure from Chomczynski and Sacchi (1987) and treated with RQ1 RNase-free DNase (Promega, Madison, WI) to elim-

inate genomic DNA contamination. RNA (50 ng), isolated as above, was subjected to one-step real-time quantitative TaqMan RT-PCR using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Gene-specific primers (CYP1a1-Mm00487218_m1; monocyte chemoattractant protein-1 (MCP1)-Mm00441242_m1; 18S-Hs99999901_s1) in the presence of TaqMan reverse transcription reagents and RT reaction mix (Applied Biosystems) were used to reverse-transcribe RNA, and TaqMan Gene Expression probes and TaqMan Universal PCR Master Mix (Applied Biosystems) were used for PCR amplification. The 18S was used as the reference gene. After an 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 at 95°C for 15 s and 40 cycles at 60°C for 1 min. The $\Delta\Delta C_t$ method was used to calculate the fold change in mRNA expression: $\Delta C_t = C_t$ (target gene) – C_t (reference gene), $\Delta\Delta C_t = \Delta C_t$ (treatment) – ΔC_t (control), fold change = $2^{(-\Delta\Delta C_t)}$ (Jiang et al., 2004).

Lung Weight/Body Weight Ratio. The mice were weighed immediately after being anesthetized, and the lungs were weighed after the sacrifice and harvesting. LW/BW ratios were determined to evaluate the severity of lung edema.

Preparation of Tissues for Histology and Immunohistochemistry. Tracheotomy was performed on the anesthetized mice, and the lung tissue was fixed by intratracheal instillation of 10% zinc formalin at a constant pressure of 25 cm of H₂O (Couroucl et al., 2002). Samples were left in solution for 24 h in formaldehyde, and then transferred to 70% ethanol for long-term storage.

Lung Histopathology. Routine histology was performed on lung tissues from individual animals after staining of the paraffin sections with hematoxylin and eosin.

Lung Immunohistochemistry for CYP1A1 Protein Expression, MCP-1 Expression, and Neutrophil Infiltration. Deparaffinized lung sections (5 μ M) were immunostained with the following primary antibodies: CD5 monoclonal CYP1A1/2 antibody (generous gift from Dr. Paul E. Thomas; dilution 1:50) for CYP1A1/2; goat polyclonal MCP-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; dilution 1:50) for MCP-1; and rat anti-mouse neutrophil antibody (Serotec, Raleigh, NC; dilution 1:200) for neutrophils, followed by staining with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). To analyze the degree of pulmonary neutrophil infiltration, the positively stained cells were counted in 10 nonadjacent areas per mouse under 40 $\times$ magnification. Dr. Roberto Barrios, a pulmonary pathologist at The Methodist Hospital Research Institute (Houston, TX), evaluated the histopathology and immunohistochemistry slides. Dr. Barrios was blinded to the treatment of mice with various regimens.

Analyses of Data. The results were analyzed by a computerized statistical package (SPSS version 19; SPSS Inc., Chicago, IL). Data are expressed as means $\pm$ S.E.M. Analysis of variance was used to assess the effects of treatment, exposure, and genotype and the interactions among them. Tukey's test was used for post hoc analysis. *P* values <0.05 were considered significant.

Results

In this investigation, we tested the hypothesis that omeprazole attenuates hyperoxic lung injury in adult WT C57BL/6J mice by an AhR-mediated induction of pulmonary and hepatic CYP1A enzymes.

Hyperoxia Attenuates Pulmonary and Hepatic CYP1A Enzyme Activities in WT Mice. The activities of EROD and MROD, which are selective for CYP1A1 and CYP1A2 enzyme, respectively, were analyzed to evaluate the effects of hyperoxia on pulmonary CYP1A1 and hepatic CYP1A1 and CYP1A2 enzyme activities. Exposure of WT animals to hyperoxia decreased pulmonary CYP1A1 ($F_{2,9} = 17.05$; $p < 0.01$; Table 1) and hepatic CYP1A2 ($F_{2,9} = 23.99$; $p < 0.01$; Table 1) enzyme activities in a time-dependent manner.

TABLE 1

Quantitative effects of hyperoxia on pulmonary and hepatic CYP1A enzyme activities

Pulmonary CYP1A1 (EROD), hepatic CYP1A1 (EROD), and hepatic CYP1A2 (MROD) activities were measured by fluorimetry in WT mice exposed to room air or hyperoxia for up to 72 h. Data are expressed as means $\pm$ S.E.M. from at least four individual animals. Significant differences between room air and hyperoxia groups are indicated (*, $P < 0.05$; **, $P < 0.01$).

Exposure	Lung CYP1A1 Activity	Liver CYP1A1 Activity	Liver CYP1A2 Activity
		<i>pmol/min/mg</i>	
Room air	4.16 $\pm$ 0.25	16.3 $\pm$ 0.76	35.05 $\pm$ 2.6
48-h Hyperoxia	3.4 $\pm$ 0.06*	14.9 $\pm$ 0.95	26.7 $\pm$ 1.48*
72-h Hyperoxia	2.9 $\pm$ 0.07**	14.17 $\pm$ 0.83	17.35 $\pm$ 0.88**

However, hepatic CYP1A1 ($F_{2,9} = 1.63$; $p = 0.249$; Table 1) enzyme activities did not change significantly upon exposure to hyperoxia.

Omeprazole Enhances Pulmonary CYP1A1 and Hepatic CYP1A2 Enzyme Activities in WT Mice. Omeprazole-treated WT mice showed increased pulmonary EROD ($F_{7,24} = 119.88$; $p < 0.01$; Fig. 1A), but not hepatic EROD ($F_{7,24} = 2.16$; $p = 0.08$; Fig. 1B) activities, compared with the corn oil group in both room air and hyperoxic conditions. Omeprazole also induced hepatic MROD activities in both room air and hyperoxic conditions in the WT mice ($F_{7,24} = 50.17$; $p < 0.01$; Fig. 1C). Hyperoxia for 72 h decreased CYP1A enzyme activities in WT mice treated with both corn oil and omeprazole. However, CYP1A enzyme activities were significantly greater in hyperoxia-exposed animals treated with omeprazole compared with room air-breathing animals treated with corn oil.

Omeprazole Enhances Pulmonary CYP1A1 and Hepatic CYP1A2 Protein Expression in WT Mice. Next, we determined the effect of omeprazole on pulmonary and hepatic CYP1A apoprotein expression. Western blot assay revealed that omeprazole increased pulmonary CYP1A1 ($F_{7,24} = 221.07$; $p < 0.01$; Fig. 2, A and B) and hepatic CYP1A2 ($F_{7,24} = 56.26$; $p < 0.01$; Fig. 2, C and D) apoprotein expression in the microsomes of WT mice exposed to room air as well as hyperoxia. It is noteworthy that hyperoxia by itself increased CYP1A apoprotein expression in both corn oil- and omeprazole-treated animals compared with their corresponding room air groups. To determine the expression of CYP1A1 protein in specific regions of the lung, we performed immunohistochemistry on fixed lung sections using CYP1A1 antibodies. Immunohistochemistry showed that omeprazole up-regulated CYP1A1 expression, as evident by enhanced positive CYP1A1 staining, in both the bronchial (Fig. 2E) and alveolar epithelium (data not shown) in WT mice exposed to room air as well as hyperoxia. These findings closely correlated with the lung Western blot assay results.

Omeprazole Enhances Pulmonary CYP1A1 and Hepatic CYP1A2 mRNA Expression in WT Mice. To determine whether the enhancement of CYP1A enzyme activities and protein contents were preceded by an increase in its mRNA, we performed real-time RT-PCR analysis from total RNA isolated from WT mice exposed to room air and hyperoxia. Quantification of mRNA levels demonstrated that omeprazole significantly increased pulmonary CYP1A1 ($F_{7,24} = 102.52$; $p < 0.01$; Fig. 3A) and hepatic CYP1A2 ($F_{7,24} = 75.41$; $p < 0.01$; Fig. 3C) mRNA levels compared with corn oil groups in both room air and hyperoxic conditions. These results were consistent with the effects of omeprazole on

Fig. 1. Omeprazole induces pulmonary and hepatic CYP1A enzyme activities via the AhR. WT and AhRd mice were pretreated with corn oil or omeprazole and exposed to room air or hyperoxia for 72 h, as described under *Materials and Methods*, after which the pulmonary EROD (CYP1A1) (A), hepatic EROD (CYP1A1) (B), and hepatic MROD (CYP1A2) (C) activities were measured by fluorimetry. Values are means $\pm$ S.E.M. from at least four individual animals in corn oil (empty bars) and omeprazole (filled bars) groups. Significant differences between corn oil and omeprazole groups are indicated by **, $p < 0.01$. Significant differences between corresponding room air and hyperoxia groups are indicated by †, $p < 0.05$.

pulmonary CYP1A1 (EROD) and hepatic CYP1A2 (MROD) enzyme activities. Omeprazole failed to induce hepatic CYP1A1 expression at the enzyme ($F_{7,24} = 1.25$; $p = 0.317$; Fig. 1B) and mRNA ($F_{7,24} = 2.16$; $p = 0.08$; Fig. 3B) levels.

Omeprazole Fails to Enhance Pulmonary and Hepatic CYP1A Expression in AhRd Mice. To examine whether AhR regulates omeprazole-mediated expression of pulmonary and hepatic CYP1A enzymes, we studied AhRd mice using the same experimental design. It is noteworthy that we found that omeprazole failed to enhance pulmonary and hepatic CYP1A expression at the enzyme (Fig. 1), protein (Fig. 2), and mRNA levels (Fig. 3) in AhRd mice exposed to both room air and hyperoxia. These results indicate that AhR is a critical regulator of omeprazole-mediated expression of pulmonary and hepatic CYP1A enzymes in mice.

Omeprazole Decreased Lung Edema, Perivascular, and Alveolar Damage in WT Mice. To estimate the severity of lung injury, we initially analyzed the LW/BW ratio to determine the degree of lung edema. The LW/BW ratio did not differ between omeprazole and corn oil groups in room air-breathing WT mice (Fig. 7A). Hyperoxia caused an increase in LW/BW ratio in WT mice compared with the corresponding room air groups. However, omeprazole attenuated hyperoxia-induced increase in the LW/BW ($P < 0.005$) ratio in WT mice compared with the corn oil group ($F_{7,24} = 107.93$; $p < 0.01$; Fig. 7A). Furthermore, histopathological examination of the lungs exposed to hyperoxia revealed less perivascular edema, alveolar hemorrhage, and infiltrates in WT mice treated with omeprazole compared with those treated with corn oil (Fig. 4). In room air-breathing WT mice, the histopathological examination of the lungs did not show any evidence of tissue injury in either the omeprazole or corn oil groups (Fig. 4).

Omeprazole Attenuates Hyperoxia-Induced Lung Inflammatory Response in WT Mice. We performed real-time RT-PCR analysis of lung MCP-1 mRNA and immunohistochemistry on fixed lung sections using anti-MCP-1 and antineutrophil antibodies to ascertain whether omeprazole altered hyperoxia-induced lung inflammatory response. Real-time RT-PCR analysis and immunohistochemistry studies revealed that hyperoxia increased both the accumulation of neutrophils (Fig. 5 and see Fig. 7B) and MCP-1 expression (Figs. 6 and 7C) in the lungs of WT mice. However, the lungs of omeprazole-treated WT mice had decreased accumulation of neutrophils (Figs. 5 and 7B; $F_{7,24} = 22.31$; $p < 0.05$) and decreased MCP-1 expression (Figs. 6 and 7C; $F_{7,24} = 181.99$; $p < 0.01$) compared with corn oil-treated WT mice exposed to hyperoxia.

Omeprazole Failed to Decrease Hyperoxia-Induced Lung Edema, Perivascular and Alveolar Damage, and Inflammation in AhRd Mice. Omeprazole failed to decrease lung edema (Fig. 7A), alveolar and perivascular damage (Fig. 4), neutrophil infiltration (Figs. 5 and 7B), and MCP-1 expression (Figs. 6 and 7C) in AhRd mice exposed to hyperoxia. In room air-breathing AhRd mice, histopathology (Fig. 4), immunohistochemistry (Figs. 5 and 6), and real-time RT-PCR (Fig. 7C) did not show any evidence of lung injury and inflammation in either the omeprazole or corn oil groups.

Discussion

In this study, we have shown that omeprazole attenuates hyperoxic lung injury in mice by an AhR-mediated induction of pulmonary CYP1A1 and hepatic CYP1A2 enzymes. In WT mice, omeprazole-mediated protection against hyperoxic lung injury correlated with enhanced pulmonary CYP1A1

Fig. 2. Omeprazole increases pulmonary and hepatic CYP1A protein expression by an AhR-dependent mechanism. A and C, representative Western blot assays showing pulmonary CYP1A1 (A) and hepatic CYP1A2 (C) apoprotein expression in microsomes isolated from WT and AhRd mice. Actin, tubulin, or glyceraldehyde-3-phosphate dehydrogenase were not used to determine protein loading because they are whole cell or cytoplasmic housekeeping proteins that might not accurately reflect equal protein loading in the microsomal fraction of the cells. CO, corn oil; OM, omeprazole. B, densitometric analysis of pulmonary CYP1A1 immunoblots. D, densitometric analysis of hepatic CYP1A2 immunoblots. Open bars: corn oil group; closed bars: omeprazole group ($n =$ at least four mice per group). Significant differences between corn oil and omeprazole groups are indicated by **, $p < 0.01$. Significant differences between corresponding room air and hyperoxia groups are indicated by †, $p < 0.05$ and ††, $p < 0.01$. E, effects of omeprazole on pulmonary CYP1A1 protein expression in WT and AhRd mice as determined by immunohistochemistry ($n =$ five mice per group). Arrows point to golden brown staining of CYP1A1-positive epithelial cells lining the bronchioles in WT mice and lack of staining in AhRd mice. Scale bar, 10 mm.

and hepatic CYP1A2 expression by omeprazole compared with control. It is noteworthy that in AhRd mice the lack of omeprazole-mediated protective effects against hyperoxic lung injury correlated with attenuated pulmonary CYP1A1 and hepatic CYP1A2 expression by omeprazole.

Omeprazole-mediated increase in pulmonary EROD and hepatic MROD activities in WT mice indicates induction of pulmonary CYP1A1 and hepatic CYP1A2 expression, because EROD and MROD activities are relatively specific for CYP1A1 and CYP1A2 enzymes, respectively (Burke et al., 1994; Moorthy et al., 2000; Courouclis et al., 2002). Enhanced pulmonary CYP1A1 and hepatic CYP1A2 mRNA expression that is seen in parallel with corresponding increases in enzyme activities provides evidence that omeprazole induces pulmonary CYP1A1 and hepatic CYP1A2 expression by transcriptional activation of CYP1A1 and CYP1A2 gene expression in WT mice. Wei et al. (2002) also observed that omeprazole induces pulmonary CYP1A1 in human lung samples using an explant culture system. Although hyperoxia decreased pulmonary and hepatic CYP1A enzyme activities and mRNA expression compared with their corresponding room air groups, CYP1A activities and mRNA expression were higher in omeprazole-treated animals exposed to hyper-

oxia compared with room air-breathing, corn oil-treated animals. These findings suggest that in hyperoxic conditions omeprazole increased CYP1A activities compared with the constitutational expression seen in room air conditions. In contrast to CYP1A enzyme activities and mRNA expression, hyperoxia increased pulmonary and hepatic CYP1A apoprotein concentrations in both the corn oil and omeprazole groups, which indicates that omeprazole and hyperoxia may affect the CYP1A apoprotein concentrations independently. The discrepancies in our observations suggest post-translational mechanisms (e.g., protein stabilization), although the exact mechanisms are unknown at this time.

Our previous experiments in mice with the prototypical CYP1A inducers 3-MC and BNF revealed that CYP1A1, but not CYP1A2, is induced in the mouse lungs (Moorthy, 2008). Therefore, we analyzed only pulmonary CYP1A1 expression in the current study. We showed that hyperoxic pulmonary toxicity is increased in mice lacking the *cyp1a2* gene (Moorthy, 2008). Another group of investigators (Shertzer et al., 2004) also observed that reactive oxygen species formation was increased in *cyp1a2*($-/-$) mice, which suggests that CYP1A2 may have antioxidant activity. These observations indicate that although CYP1A2 is mainly hepatic it can have

Fig. 3. Real-time RT-PCR analysis showing the effects of omeprazole on pulmonary CYP1A1 (A), hepatic CYP1A1 (B), and hepatic CYP1A2 (C) mRNA expression in WT and AhRd mice. Values are means $\pm$ S.E.M. from at least four individual animals in corn oil (empty bars) and omeprazole (filled bars) groups. Significant differences between corn oil and omeprazole groups are indicated by *, $p < 0.05$ and **, $p < 0.01$. Significant differences between corresponding room air and hyperoxia groups are indicated by †, $p < 0.05$ and ††, $p < 0.01$.

extrahepatic protective effects against hyperoxic lung injury. CYP1A2 is mainly a hepatic enzyme and is rarely expressed in extrahepatic tissues. Although CYP1A1 enzymes are also expressed in the intestines and kidneys, the effects of intestinal and renal CYP1A1 on hyperoxic lung injury are unknown. Therefore, we analyzed the effects of omeprazole on

Fig. 4. Omeprazole decreases hyperoxia-induced lung edema and perivascular and alveolar injury by an AhR-dependent mechanism. Representative hematoxylin/eosin-stained images from the lungs of WT and AhRd mice ($n =$ five mice per group) are shown. a, corn oil-treated WT mice exposed to room air. b, corn oil-treated WT mice exposed to hyperoxia. c, omeprazole-treated WT mice exposed to room air. d, omeprazole-treated WT mice exposed to hyperoxia. e, corn oil-treated AhRd mice exposed to room air. f, corn oil-treated AhRd mice exposed to hyperoxia. g, omeprazole-treated AhRd mice exposed to room air. h, omeprazole-treated AhRd mice exposed to hyperoxia. Arrows and arrowheads point to perivascular and alveolar areas, respectively. Scale bar, 10 μ m.

hepatic CYP1A2 in addition to pulmonary CYP1A1 in the current study.

The mechanistic role of AhR in the induction of CYP1A by prototypical inducers, 3 MC and BNF, has been extensively studied. However, the molecular mechanism of induction of CYP1A by omeprazole remains obscure. Therefore, we conducted experiments with omeprazole in mice having a dysfunctional AhR to delineate the precise role of AhR in omeprazole-mediated induction of CYP1A enzymes. In AhRd mice, the failure of omeprazole to enhance pulmonary EROD and hepatic MROD activities and CYP1A apoprotein, protein, and mRNA expression in both room air and hyperoxia supports the hypothesis that the induction of pulmonary CYP1A1 and hepatic CYP1A2 by omeprazole is mediated by AhR-dependent mechanisms. Our observation that AhR is critical for the up-regulation of CYP1A gene by omeprazole

Fig. 5. Representative immunostained images for neutrophils in the lungs. Effects of omeprazole on hyperoxia-induced neutrophil recruitment into the lungs was determined by immunohistochemistry with antineutrophil antibodies in WT and AhRd mice ($n =$ five mice per group). a, corn oil-treated WT mice exposed to room air. b, corn oil-treated WT mice exposed to hyperoxia. c, omeprazole-treated WT mice exposed to room air. d, omeprazole-treated WT mice exposed to hyperoxia. e, corn oil-treated AhRd mice exposed to room air. f, corn oil-treated AhRd mice exposed to hyperoxia. g, omeprazole-treated AhRd mice exposed to room air. h, omeprazole-treated AhRd mice exposed to hyperoxia. Arrows point to brown staining neutrophils in the lungs. Scale bar, 10 μ m.

is consistent with other studies (Denison and Nagy, 2003; Yoshinari et al., 2008).

The dose of omeprazole used in this study was comparable with the dose used in previous studies in rodents (Larsson et al., 1988; Kashfi et al., 1995). Numerous studies have shown clear differences in the induction of CYP1A by omeprazole among the species examined (Shih et al., 1999). Omeprazole seems to be a more potent inducer of CYP1A in humans than in rodents and has been shown to induce CYP1A in humans with conventional doses used to treat gastric acid related disorders (Kashfi et al., 1995; Shih et al., 1999). Rodents require a considerably higher dose of omeprazole than humans to induce CYP1A. Differential mechanisms by which omeprazole enhances CYP1A gene transcription may be responsible for the differences observed among species (Tompkins and Wallace, 2007).

Fig. 6. Omeprazole decreases hyperoxia-induced expression of MCP-1 in the lungs of WT mice. Representative immunostained images for MCP-1 expression in the lungs of WT and AhRd mice ($n =$ four mice per group) are shown. a, corn oil-treated WT mice exposed to room air. b, corn oil-treated WT mice exposed to hyperoxia. c, omeprazole-treated WT mice exposed to room air. d, omeprazole-treated WT mice exposed to hyperoxia. e, corn oil-treated AhRd mice exposed to room air. f, corn oil-treated AhRd mice exposed to hyperoxia. g, omeprazole-treated AhRd mice exposed to room air. h, omeprazole-treated AhRd mice exposed to hyperoxia. Arrows point to the brown staining of MCP-1-positive epithelial cells lining the bronchioles. Scale bar, 10 μ m.

Our study demonstrates that omeprazole attenuates hyperoxia induced: 1) alveolar and perivascular damage (Fig. 4) and 2) inflammation (Figs. 5–7) in WT mice. Attenuation of lung injury in omeprazole-treated WT mice exposed to hyperoxia signifies the protective effects of omeprazole against hyperoxic lung injury. Our observation that omeprazole fails to protect against hyperoxic lung injury in AhRd mice suggests that AhR plays a crucial role in omeprazole-mediated protection against hyperoxic lung injury. Because the AhR regulates the induction of CYP1A enzymes that may detoxify reactive oxygen species (Tong et al., 2003; Sinha et al., 2005; Moorthy, 2008), it is possible that suppression of these enzymes may have contributed to the failure of omeprazole to protect AhRd mice against hyperoxic lung injury. The protective effects of CYP1A enzymes against hyperoxic lung injury in rodents have been extensively documented, as evi-

Fig. 7. Representative quantitative analysis of the effects of omeprazole on hyperoxia-induced lung injury and inflammation. A, effects of omeprazole on lung weight/body weight ratios of WT and AhRd mice. Values are means $\pm$ S.E.M. from at least four individual animals in corn oil (empty bars) and omeprazole (filled bars) groups. Significant differences between corn oil and omeprazole groups are indicated by **, $p < 0.01$. Significant differences between corresponding room air and hyperoxia groups are indicated by ††, $p < 0.01$. B, neutrophil count analysis per high-power

denced by 1) attenuation of hyperoxic lung injury in rodents treated with CYP1A inducers, BNF or 3-MC (Mansour et al., 1988; Sinha et al., 2005; Moorthy, 2008); 2) potentiation of hyperoxic lung injury in rats treated with CYP1A inhibitor, 1-aminobenzotriazole (Moorthy et al., 2000); and 3) increased susceptibility of rodents deficient in the genes for AhR (Coudrouli et al., 2002; Jiang et al., 2004) to hyperoxic lung injury. Because AhR also induces phase II enzymes such as NAD(P)H quinone reductase, glutathione transferase- α , and aldehyde dehydrogenase, the beneficial roles of these enzymes against hyperoxic lung injury are not excluded. It is also possible that superoxide dismutase may have contributed to some of the beneficial effects of the AhR, which may up-regulate superoxide dismutase through the Ah-responsive elements (Park and Rho, 2002).

It has been postulated that the antiulcer and gastroprotective effects of omeprazole may also involve acid-unrelated mechanisms, which include inhibition of neutrophil infiltration and oxidative tissue damage (Kobayashi et al., 2002; Pozzoli et al., 2007). To support this, several in vitro studies have revealed that omeprazole possesses a direct scavenging activity against oxygen free radicals and inhibits neutrophil function (Wandall, 1992; Yoshida et al., 2000). Thus, it is plausible that the antioxidant properties of omeprazole may be beneficial in other pathologic conditions associated with oxidative damage (Halliwell et al., 2000; Hanauer, 2006). Omeprazole has also been shown to protect against necrotizing enterocolitis in newborn rats subjected to hypoxia/reoxygenation (Cadiri et al., 2008). All of these data show that omeprazole has antioxidant and anti-inflammatory properties. However, the mechanisms of the antioxidant and anti-inflammatory effects of omeprazole are not clear. In the current study, although we have not provided a direct link between omeprazole, CYP1A induction, and hyperoxic lung injury, we have clearly shown that omeprazole-mediated attenuation of hyperoxic lung injury observed histopathologically was associated with enhanced pulmonary CYP1A1 and hepatic CYP1A2 expression in WT mice. On the contrary, omeprazole failed to protect against hyperoxic lung injury in AhRd mice, and these mice were refractory to the induction of CYP1A enzymes by omeprazole. These results support the concept that omeprazole protects against hyperoxic lung injury via AhR-dependent mechanisms and is also associated with the induction of CYP1A enzymes by omeprazole. To our knowledge, this is the first study to report a novel finding that omeprazole protects against hyperoxic lung injury in mice.

In summary, we provide evidence that omeprazole therapy attenuates hyperoxic injury in mice in vivo by inducing pulmonary CYP1A1 and hepatic CYP1A2 enzymes via an AhR-mediated mechanism. We propose that the protective effects

field. Data represent means $\pm$ S.E.M. from at least four individual animals in corn oil (empty bars) and omeprazole (filled bars) groups. Significant differences between corn oil and omeprazole groups are indicated by*, $p < 0.05$. Significant differences between corresponding room air and hyperoxia groups are indicated by ††, $p < 0.01$. C, real-time RT-PCR analysis showing the effects of omeprazole on pulmonary MCP-1 mRNA expression in WT and AhRd mice. Values are means $\pm$ S.E.M. from at least four individual animals in corn oil (empty bars) and omeprazole (filled bars) groups. Significant differences between corn oil and omeprazole groups are indicated by **, $p < 0.01$. Significant differences between corresponding room air and hyperoxia groups are indicated by ††, $p < 0.01$.

of omeprazole may be caused by pulmonary CYP1A1 and hepatic CYP1A2-mediated detoxification of lipid peroxides and hydroperoxides generated by reactive oxygen species. Our results suggest that omeprazole may be beneficial as an adjunctive therapeutic agent in the prevention and treatment of hyperoxia-induced disorders such as BPD in premature infants and acute respiratory distress syndrome in older children and adults. Further studies are needed to investigate the safety and efficacy of omeprazole against hyperoxic lung injury in neonatal mice in vivo and evaluate the effects of omeprazole on human infant pulmonary cell lines exposed to hyperoxia in vitro. These studies may provide additional insight into the mechanisms of protection and the feasibility of the use of omeprazole for hyperoxic lung injury in human infants.

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Authorship Contributions

Participated in research design: Shivanna, Jiang, Couroucli, and Moorthy.

Conducted experiments: Shivanna, Jiang, and Wang.

Performed data analysis: Shivanna, Jiang, and Moorthy.

Wrote or contributed to the writing of the manuscript: Shivanna and Moorthy.

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