Angiotensin II Type 1 Receptor Antagonist Attenuates Lung Fibrosis in Hyperoxia-Exposed Newborn Rats

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ABSTRACT
Bronchopulmonary dysplasia (BPD) remains a major cause of morbidity and mortality during the first year of life, and many infants have significant respiratory problems throughout childhood. Currently no effective therapy is clinically available to prevent the long-term pulmonary sequelae of BPD. Previous research has demonstrated that the renin-angiotensin system is up-regulated in human lung fibroblasts. Angiotensin II type 1 receptor (AT1R) antagonists and AT1R short interfering RNA diminished hyperoxia-increased collagen expression, whereas AT2R antagonists did not have any effects on these hyperoxia-induced changes. The in vivo therapeutic effects of AT1R antagonists on hyperoxia-induced lung fibrosis remain unknown. The present study assessed the effects of an AT1R antagonist (losartan) on preventing hyperoxia-induced lung fibrosis in newborn rats. Rat pups were exposed to 7 days of >95% O2 and an additional 2 weeks of 60% O2. AT1R antagonist-treated pups were injected intraperitoneally with losartan at a dose of 10 mg/kg/day from postnatal days 1 to 7 and a dose of 5 mg/kg/day from postnatal days 8 to 21. Control group pups were injected with an equal volume of normal saline. AT1R antagonist treatment attenuated the hyperoxia-induced lung fibrosis on postnatal days 7 and 21 and also decreased the hyperoxia-induced expression of extracellular signal-regulated protein kinase and -smooth muscle actin. AT1R antagonist treatment did not affect body weight or lung weight of the rats. These data suggest that AT1R antagonist may offer a novel therapeutic strategy for preventing hyperoxia-induced lung fibrosis.

Introduction
Bronchopulmonary dysplasia (BPD) remains a major cause of morbidity and mortality in the first year of life. Many infants have significant respiratory problems throughout childhood, including increased airway reactivity and development of obstructive airway disease (Lemons et al., 2001). Some abnormal lung functions may persist into adulthood (Northway et al., 1990). The pathogenesis of BPD is multifactorial, and researchers believe pulmonary oxygen toxicity may play an important role in the lung injury process, which leads to the development of BPD (Welty, 2001). In previous studies, prolonged exposure of neonatal mice to hyperoxia resulted in decreased alveolar septation, increased terminal air space size, and increased lung fibrosis in a similar manner to human BPD (Manji et al., 2001; Couroucli et al., 2006; Chen et al., 2007). Currently no effective therapy is clinically available to prevent long-term pulmonary sequelae of BPD. The renin-angiotensin system (RAS) is a key regulator of blood pressure and fluid homeostasis (Peach, 1977; Morrey et al., 2010). Angiotensin (Ang) II is a main effector molecule of the RAS, produced from the substrate angiotensinogen through sequential enzymatic cleavages by renin and angiotensin-converting enzyme. Tissues throughout the body synthesize various components of the RAS, which may be subject to local control (Dzau et al., 1987; Veerappan et al., 2008). A previous investigation reported high Ang II concentrations in normal rat lung (Tryka et al., 1986). Another study identified angiotensinogen and Ang II type 1 receptor (AT1R) expression in rat lung tissue (Campbell et al., 1995). Angiotensin (Ang) II is a main effector molecule of the RAS, produced from the substrate angiotensinogen through sequential enzymatic cleavages by renin and angiotensin-converting enzyme. Tissues throughout the body synthesize various components of the RAS, which may be subject to local control (Dzau et al., 1987; Veerappan et al., 2008). A previous investigation reported high Ang II concentrations in normal rat lung (Tryka et al., 1986). Another study identified angiotensinogen and Ang II type 1 receptor (AT1R) expression in rat lung tissue (Campbell et al., 1995). Ang II is a potential profibrotic mediator, inducing human lung fibroblast proliferation via activation of the AT1R and stimulating...
collagen synthesis in human lung fibroblasts (Marshall et al., 2000, 2004). We previously demonstrated that hyperoxia increased the expression of collagen and RAS components in human lung fibroblasts (Lang et al., 2010). The AT₁R antagonist losartan and AT₁R short interfering RNA diminished the hyperoxia-increased collagen expression. However, the AT₁R antagonist 1-[(4-dimethylamino)-3-methylphenyl]-methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid difluoroacetate (PD123319) did not have any effects on these hyperoxia-induced changes. The in vivo therapeutic potential of AT₁R antagonists in hyperoxia-induced lung fibrosis remains unknown. The aim of this study, therefore, was to test whether an AT₁R antagonist would attenuate hyperoxia-induced lung fibrosis in newborn rats.

**Materials and Methods**

**Animals.** This study was performed in accordance with the guidelines provided by the Animal Care Use Committee of Taipei Medical University. A total of eight time-dated pregnant Sprague-Dawley rats were housed in individual cages with free access to laboratory food and water and ad libitum, kept in a 12-h light/dark cycle, and allowed to deliver vaginally at term. Four pregnant dams were assigned to both room air and hyperoxia groups.

**Hyperoxia Exposure.** Within 12 h of birth, litters were separated from their mothers, pooled before being randomly redistributed to the newly delivered mothers, and exposed to >95% O₂ or room air (Fig. 1). Rat pups were injected intraperitoneally with losartan (Sigma-Aldrich, St. Louis, MO) at a dose of 10 mg/kg/day from postnatal days 1 to 7 and a dose of 5 mg/kg/day from postnatal days 8 to 21. The dosage of losartan was based on recommendations by Li et al. (2003). Control groups were injected with an equal volume of normal saline. Nursing mothers were rotated between oxygen-exposed and room air litters every 24 h to avoid oxygen toxicity in the mothers and eliminate maternal effects between groups. Oxygen exposures were performed in transparent 40 × 50 × 60-cm Plexiglas chambers, into which oxygen was continuously delivered at 4 l/min, and oxygen levels were monitored by using a Proox model 110 monitor (BioSpherix, Redfield, NY). Litters were exposed to 7 days of >95% O₂ and then allowed to recover from the acute injury in a continuing environment of moderate hyperoxia (60% O₂) for an additional 2 weeks (3 weeks of total hyperoxia). Body and lung weights were recorded at the time of sacrifice. Animals were killed by an intraperitoneal injection of pentobarbital sodium and exsanguinated by aortic transection. Lung tissue from room air- and hyperoxia-exposed pups was harvested on postnatal days 7 and 21.

**Real-Time Polymerase Chain Reaction.** Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized with a First-Strand cDNA Synthesis Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Primer sequences for real-time polymerase chain reaction were: collagen type I sense 5'-CAACCTCAAGAAGTCCCTGC-3', antisense 5'-AGTGGAATCGACTGTGTTGCCCT-3'; α-smooth muscle actin (α-SMA) sense 5'-GCTCTGTTGTTGAACTG-3', antisense 5'-ACGATGGAGTGAAAAACAG-3'; and GAPDH sense 5'-CTCCCTCAAGATTGTACGGACTAAG-3', antisense 5'-GTCCAGATCCACACCGGATACTAT-3'. Gene expression was quantitatively analyzed by using the comparative CT (ΔCT) method, in which CT is the threshold cycle number (the minimum number of cycles needed before the product can be detected). The arithmetic formula for the ΔCT method is the difference in threshold cycles for a target and an endogenous reference (the GAPDH housekeeping gene). The amount of target normalized to an endogenous reference and relative to a calibration normalized to an endogenous reference is given by 2ΔΔCT.

**Measurement of Total Collagen in Lung Tissue.** Lung collagen was determined by assaying total soluble collagen using the Sircol collagen assay kit (Biocolor Ltd., New Abbey, UK) according to the manufacturer’s instructions. This method measures newly synthesized collagen, which has not been extensively cross-linked. Previously published articles have demonstrated that measurements performed using the Sircol assay correlate with histological evidence of pulmonary fibrosis in an animal model (Chung et al., 2003). In brief, lungs were homogenized in 5 ml of 0.5 M acetic acid containing 1 mg of pepsin (Sigma-Aldrich) per 10 mg of tissue residue. Each sample was incubated for 24 h at 4°C with stirring. After centrifugation, 100 μl of each supernatant was assayed. One milliliter of Sircol dye reagent, which specifically binds to collagen, was then added to each sample and mixed for 30 min. After centrifugation, the pellet was suspended in 1 ml of alkali reagent (0.5 M NaOH) included in the kit, and the optical density was evaluated at 540 nm by using a spectrophotometer. Values in the test samples were compared with values obtained with collagen standard solutions provided by the manufacturer that were used to construct a standard curve.

**Western Blot Analysis.** Lung proteins were resolved by using 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Billerica, MA). The membranes were then incubated with a rabbit antil collagen type I polyclonal antibody (1:10,000; Abcam Inc., Cambridge, MA), a rabbit anti-α-SMA monoclonal antibody (1:50,000; Epitomics, Burlingame, CA), a rabbit anti-extracellular signal-regulated protein kinase (ERK) monoclonal antibody (1:2000; Cell Signaling Technology, Danvers, MA), or a mouse anti-β-actin monoclonal antibody (1:100,000; Sigma-Aldrich). After incubation with the primary antibody, the membranes were probed with horseradish peroxidase-conjugated secondary antibody (1:20,000; anti-mouse, Thermo Fisher Scientific, Waltham, MA). For Western blot analysis, the densitometry unit of the protein expression.
in room air-exposed lungs was assigned as 1 after being normalized to β-actin.

**Histological Examination.** The rat lungs were immersed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for light microscopic evaluation. The sampled tissues were dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Seven-micrometer tissue sections were stained with hematoxylin and eosin and examined by a pathologist who was blinded to the protocol and experimental groups. Five random high-power (400×) fields were evaluated for the presence of hemorrhage, alveolar septal thickness, and inflammatory cell infiltration, using a scale of 0 to 4. The macrophage numbers of each section were determined by counting five random fields at 400× magnification. The number of macrophages was averaged for each group and compared statistically. Other lung sections were stained with Masson’s trichrome and assessed for the presence of collagen. The selected sections were viewed and photographed by using a 10× objective lens and a Nikon (Tokyo, Japan) Eclipse E600 microscope. A minimum of four randomly selected fields were captured at 100× magnification for each section by using a digital camera and imported into a computerized image analysis system (Image-Pro Plus 5.1 for Windows; Media Cybernetics, Inc., Bethesda, MD). The automatic object density process was used for the quantification of collagen accumulation in the lung tissue.

**Statistical Analysis.** Results are presented as the mean ± S.D. Analysis of differences between the two groups on each postnatal day was evaluated by using one-way analysis of variance (ANOVA) with post hoc Scheffe’s test. Survival rate was evaluated by using the Kaplan-Meier method, and the log-rank test was used for comparison.

**Results**

**Body Weight, Lung Weight, and Lung/BODY Weight Ratio (%).** Tables 1 and 2 present the effects of hyperoxia and losartan treatment on body weight, lung weight, and the lung/body weight ratio (%) on postnatal days 7 and 21, respectively. Body weights and lung weights increased as rats aged. Rats exposed to hyperoxia exhibited significantly lower body weights and lower lung weights compared with room-air controls on postnatal days 7 and 21, and the values were comparable between vehicle- and losartan-treated rats. The lung/body weight ratio was smaller in hyperoxia-exposed rats on postnatal day 7 and significantly larger in hyperoxia-exposed rats compared with room-air controls on postnatal day 21. Body weight, lung weight, and the lung/body weight ratio were comparable between vehicle- and losartan-treated rats in room air and hyperoxia groups on postnatal days 7 and 21.

**TABLE 1**

Body weight, lung weight, and the lung/body weight ratio in room air- and hyperoxia-exposed rats on postnatal day 7

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Body Weight</th>
<th>Lung Weight</th>
<th>Lung/BODY Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room air + vehicle</td>
<td>6</td>
<td>11.61 ± 0.84</td>
<td>0.22 ± 0.03</td>
<td>1.87 ± 0.25</td>
</tr>
<tr>
<td>Room air + losartan</td>
<td>7</td>
<td>11.79 ± 0.89</td>
<td>0.24 ± 0.04</td>
<td>2.01 ± 0.25</td>
</tr>
<tr>
<td>Hyperoxia + vehicle</td>
<td>8</td>
<td>9.13 ± 0.84</td>
<td>0.16 ± 0.04b</td>
<td>1.77 ± 0.34</td>
</tr>
<tr>
<td>Hyperoxia + losartan</td>
<td>8</td>
<td>9.30 ± 0.41a</td>
<td>0.15 ± 0.02a</td>
<td>1.60 ± 0.17a</td>
</tr>
</tbody>
</table>

a Different from room air-exposed rats at P < 0.001.
b Different from room air-exposed rats at P < 0.01.
ap Different from room air + losartan group at P < 0.05.

**Survival.** Hyperoxia decreased survival rate, mainly on postnatal day 7, reaching 70% in the hyperoxia + vehicle group (Fig. 2). Losartan treatment increased hyperoxia-decreased survival rate to 80%. The differences in survival rate between newborn rats treated by losartan under hyperoxia and those under room air and receiving either vehicle or losartan were not significant.

**Histology.** Figure 3 presents representative lung sections from room air- and hyperoxia-exposed rats. Room air-exposed animals treated with the vehicle or losartan showed normal lung structure on postnatal day 7, and there was no evidence of tissue injury (Fig. 3A, a and b). On postnatal day 7, the lungs of the hyperoxia-exposed rats contained larger, more thin-walled air spaces compared with room air-exposed rats (Fig. 3A, a and c; Table 3). The changes in the histologic scores in the categories of hemorrhage and interstitial inflammation were not statistically significant on postnatal day 7 (Table 3). On postnatal day 21, the lungs of hyperoxia-exposed rats contained more hemorrhage and wide interstitium with inflammatory cell recruitment compared with room air-exposed animals (Fig. 3B; Table 4). Hyperoxia treatment of newborn rats significantly increased lung macrophage number compared with room air-exposed rats, and losartan treatment decreased macrophage number on postnatal days 7 and 21 (Table 3 and 4).

**Effects of Hyperoxia on Collagen I and α-SMA mRNA Expression.** Hyperoxic exposure for 7 and 21 days significantly increased collagen type I and α-SMA mRNA expression compared with room air-exposed rats (Fig. 4). The increased collagen type I and α-SMA mRNA expression...
Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described under Materials and Methods. Animals were sacrificed on postnatal day 7 (A) or 21 (B). a, room air-exposed rats treated with vehicle; b, room air-exposed rats treated with losartan; c, hyperoxia-exposed rats treated with vehicle; d, hyperoxia-exposed rats treated with losartan.

**Discussion**

In the present study's in vivo model, exposure of newborn rats to hyperoxia increased total collagen, collagen type I, and α-SMA on postnatal days 7 and 21. The significant decreases in body weight and lung weight in hyperoxic animals on postnatal days 7 and 21 (Tables 1 and 2) and the histological evidence of lung damage on postnatal day 7 (Fig. 2) were consistent with previous studies showing acute lung

**TABLE 3**

Lung histological features in room air- and hyperoxia-exposed rats on postnatal day 7

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Hemorrhage</th>
<th>Alveolar Septal Thickness</th>
<th>Interstitial Inflammation</th>
<th>Macrophage/Lung Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room air + vehicle</td>
<td>6</td>
<td>0.0 ± 0.0</td>
<td>1.5 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td>7.3 ± 1.3</td>
</tr>
<tr>
<td>Room air + losartan</td>
<td>7</td>
<td>0.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td>Hyperoxia + vehicle</td>
<td>8</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.1*</td>
<td>0.0 ± 0.1</td>
<td>20.8 ± 4.3*</td>
</tr>
<tr>
<td>Hyperoxia + losartan</td>
<td>8</td>
<td>0.0 ± 0.0</td>
<td>0.8 ± 0.3*</td>
<td>0.0 ± 0.0</td>
<td>8.0 ± 1.4</td>
</tr>
</tbody>
</table>

a Different from room air-exposed rats at \( P < 0.001 \).  
b Different from room air-exposed rats and hyperoxia + losartan group at \( P < 0.001 \).

**TABLE 4**

Lung histological features in room air- and hyperoxia-exposed rats on postnatal day 21

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Hemorrhage</th>
<th>Alveolar Septal Thickness</th>
<th>Interstitial Inflammation</th>
<th>Macrophage/Lung Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room air + vehicle</td>
<td>6</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>8.5 ± 1.0</td>
</tr>
<tr>
<td>Room air + losartan</td>
<td>6</td>
<td>0.0 ± 0.0</td>
<td>1.2 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>8.0 ± 1.8</td>
</tr>
<tr>
<td>Hyperoxia + vehicle</td>
<td>6</td>
<td>0.3 ± 0.3*</td>
<td>1.0 ± 0.4</td>
<td>0.2 ± 0.3</td>
<td>13.8 ± 2.3*</td>
</tr>
<tr>
<td>Hyperoxia + losartan</td>
<td>8</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>8.8 ± 2.0</td>
</tr>
</tbody>
</table>

a Different from room air-exposed rats and hyperoxia + losartan group at \( P < 0.05 \).  
b Different from room air-exposed rats and hyperoxia + losartan group at \( P < 0.01 \).
The main finding of this study is that the AT1R antagonist losartan attenuated hyperoxia-induced lung fibrosis in newborn rats. The results suggest that AT1R plays a significant role in the pathogenesis of hyperoxia-induced lung fibrosis.

AT1R antagonist at the dose of 7.5 mg/kg for 8 weeks did not influence systolic blood pressure in the normotensive rats, and losartan at a dose of 10 mg/kg completely prevented collagen gene activation and attenuated the degree of renal vascular fibrosis without influencing the systolic blood pressure in the transgenic mice (Takemoto et al., 1997; Boffa et al., 1999). Other potential actions by which AT1R antagonists might act on the lung include decreased vascular tone, decreased vascular permeability, and altered fibroblast activity (Marshall, 2003). Thus, it is probable that antifibrotic action of losartan might be related to lower systemic blood pressure. However, blood pressure was not measured in the present study, and the data do not exclude that possibility.

The alveolar stage of lung development in the mouse begins on postnatal day 4, and saccular division is completed by postnatal day 14 (Burri, 1974). The newborn rat is particularly appropriate for studies of neonatal oxygen injury because the developmental stage of the rodent lung at birth overlaps with that of the human preterm neonate at 24 to 28 weeks gestation (Han et al., 1996). During the study’s 3-week period of hyperoxia, the body weight of the hyperoxia-exposed rats reduced to ~80 and ~35% of the body weight of the room air-exposed rats on postnatal days 7 and 21, respectively (Tables 1 and 2).

The lung weight of hyperoxia-exposed rats also significantly decreased compared with the room-air groups. In hyperoxia-exposed rats, loss of body weight was proportionally greater than lung weight loss, resulting in an increased lung to body weight ratio on postnatal day 21. The trends in changes to body weight and lung weight are similar to our group’s previous hyperoxia study (Chen et al., 2007). Body weight, lung weight, and the lung/body weight ratio were comparable between vehicle- and losartan-treated rats in room air and hyperoxia groups on postnatal days 7 and 21. These results indicate that losartan treatment had no effects on body weight, lung weight, or the lung/body weight ratio of room air- or hyperoxia-exposed rats.
Hyperoxia-induced lung injury is characterized by the death of cells. Increased proliferation and differentiation of fibroblasts replaces these cells (Tryka et al., 1986). Failure to modify these processes may lead to excessive cell growth and proliferation, collagen overproduction, and pulmonary fibrosis. Collagen is the major extracellular matrix component of the lungs and vital for maintaining normal lung architecture. Collagen type I is the most abundant collagen subtype in normal human lungs (Kirk et al., 1984). Pulmonary fibrosis is the final result of hyperoxia-induced lung injury and is characterized by fibroblast proliferation and differentiation to myofibroblasts, which are responsible for the production of the extracellular matrix (Rehan and Torday, 2003). Myofibroblasts are the predominant source of collagen type I, have a phenotypic intermediate between fibroblasts and smooth muscle cells, and are defined by the presence of α-SMA (Tomasak et al., 2002). This study identified elevated collagen type I and α-SMA expressions after hyperoxic treatment (Fig. 5). These findings parallel those of myofibroblast formation studies and link the expression of the myofibroblast phenotype to the elevation of collagen synthesis.

Masson's trichrome differentiated collagen from smooth muscle and elastin and better visualized and quantified the extent of airway fibrosis. After Masson's trichrome staining, collagen presented as a dense bluish-tinged material, as shown surrounding the small and large airways and perivascular interstitium in Fig. 4. Increased collagen deposition in the interstitium and interalveolar septum occurred in hyperoxic-exposed rats compared with room air-exposed rats on postnatal days 7 and 21 (Fig. 4). In contrast, hyperoxia-exposed and losartan-treated rats exhibited significantly less collagen deposition compared with room air- and hyperoxia-exposed and vehicle-treated rats.

This study demonstrated that hyperoxia increased p-ERK expression (Fig. 5). Mitogen-activated protein kinases (MAPKs) act as signal transducers in response to cellular stresses. A prior investigation linked ERK and p38 MAPK to AT_{1}R activation (Li et al., 2005). The group's previous study demonstrated that ERK1/2, but not p38 MAPK, is involved in the hyperoxia-induced increase of collagen type I in human lung fibroblasts (Lang et al., 2010). The present in vivo study identified augmentation of ERK1/2 subsequent to hyperoxic exposure on postnatal days 7 (a ~2-fold increase) and 21 (a ~4-fold increase). The AT_{1}R antagonist losartan decreased the hyperoxia-induced ERK1/2 protein expression. These re-
results suggest the involvement of ERK1/2 in hyperoxia-induced lung fibrosis.

In conclusion, this study further confirms the functional significance of RAS signaling in the pathogenesis of hyperoxia-induced lung injury and the therapeutic potential of AT,R antagonists in protecting against hyperoxia-induced lung fibrosis. This study also reveals that an AT,R antagonist does not have deleterious effects on normal neonatal lung development. Currently no effective therapy is clinically available to prevent hyperoxia-induced lung fibrosis. AT,R antagonist therapy may provide a novel strategy for preventing hyperoxia-induced lung fibrosis.

Authorship Contributions

Participated in research design: Chou, Wang, and Chen.

Conducted experiments: Chou, Lang, Wang, Wu, Hsieh, and Chen.

Performed data analysis: Chou, Lang, Wang, Wu, and Chen.

Wrote or contributed to the writing of the manuscript: Chou and Chen.

References


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