JPET Fast Forward. Published on October 17, 2011 as DOI: 10.1124/jpet.111.186288
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JPET #186288

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

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Running title: AT_1R Antagonist Attenuates Hyperoxia-Induced Lung Fibrosis

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The number of text pages: 25 pages

The number of tables: 4 tables

The number of figures: 7 figures

The number of and references: 26 references

The number of words in the Abstract: 236 words

The number of words in the Introduction: 353 words

The number of words in the Discussion: 965 words

ABBREVIATIONS: BPD, bronchopulmonary dysplasia; RAS, renin-angiotensin system; Ang, angiotensin; AT_1R, Angiotensin II type 1 receptor; α-SMA, alpha-smooth muscle actin; ERK, extracellular signal-regulated protein kinase; ANOVA, analysis of variance; MAPK, mitogen-activated protein kinase.

A recommended section assignment: Toxicology

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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 (RAS) is upregulated in human lung fibroblasts. Angiotensin II type 1 receptor (AT_1R) antagonists and AT_1R siRNA diminished hyperoxia-increased collagen expression, while AT_2R antagonists did not have any effects on these hyperoxia-induced changes. The *in vivo* therapeutic effects of AT_1R antagonists on hyperoxia-induced lung fibrosis remain unknown. The present study assessed the effects of an AT_1R antagonist (losartan) on preventing hyperoxia-induced lung fibrosis in newborn rats. Rat pups were exposed to seven days of >95 % O_2 and a further two weeks of 60 % O_2. AT_1R antagonist-treated pups were injected intraperitoneally with losartan at a dose of 10 mg/kg/day from postnatal days one to seven, and 5 mg/kg/day from postnatal days eight to 21. Control group pups were injected with an equal volume of normal saline. AT_1R antagonist treatment attenuated the hyperoxia-induced lung fibrosis on postnatal days seven and 21, and also decreased the hyperoxia-induced expression of extracellular signal-regulated protein kinase and alpha-smooth muscle actin. AT_1R antagonist treatment did not affect body weight or lung weight of the rats. These data suggest that AT_1R antagonist may offer a novel therapeutic strategy to prevent

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hyperoxia-induced lung fibrosis.

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Introduction

Bronchopulmonary dysplasia (BPD) remains a major cause of morbidity and mortality during the first year of life. Many infants have significant respiratory problems throughout childhood, including increased airway reactivity and development of obstructive airway disease (Lemons, 1996). Some abnormal lung functions may persist into adulthood (Northway, 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, 1987; Veerappan et al., 2008). A previous investigation reported high Ang II concentrations in normal rat lung (Tryka, 1986). Another prior study identified angiotensinogen and Ang II

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type 1 receptor (AT_1R) expression in rat lung tissue (Campbell, 1995). Ang II is a potential profibrotic mediator, inducing human lung fibroblast proliferation via activation of the AT_1R and stimulating collagen synthesis in human lung fibroblasts (Marshall et al., 2000; Marshall et al., 2004). The present group previously demonstrated that hyperoxia increased the expression of collagen and RAS components in human lung fibroblasts (Lang et al., 2010). The AT_1R antagonist losartan and AT_1R siRNA diminished the hyperoxia-increased collagen expression. However, the AT_2R antagonist PD123319 did not have any effects on these hyperoxia-induced changes. The *in vivo* therapeutic potential of AT_1R antagonists in hyperoxia-induced lung fibrosis remains unknown. The aim of this study, therefore, was to test if an AT_1R 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 *ad libitum*, kept in a 12:12 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 % O_2 or room air (Fig. 1). Rat pups were injected intraperitoneally with losartan

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(Sigma-Aldrich, St. Louis, MO, USA) at a dose of 10 mg/kg/day from postnatal days one to seven, and 5 mg/kg/day from postnatal days eight 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 to eliminate maternal effects between groups. Oxygen exposures were performed in transparent 40 x 50 x 60 cm Plexiglas chambers, into which oxygen was continuously delivered at 4 l/min and oxygen levels were monitored using a Pro:ox Model 110 monitor (BioSpherix, Redfield, NY, USA). Litters were exposed to seven days of >95 % O_2 and then allowed to recover from the acute injury in a continuing environment of moderate hyperoxia (60 % O_2) for a further two weeks (three 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 were exsanguinated by aortic transection. Lung tissue from room air- and hyperoxia-exposed pups was harvested on postnatal days seven and 21.

Real-Time Polymerase Chain Raction (PCR). Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized with a First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ, USA). Primer sequences for real-time PCR were collagen type I sense 5'-CAACCTCAAGAAGTCCCTGC-3', antisense 5'-AGGTGAATCGACTGTTGCCT-3';

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alpha smooth muscle actin (α-SMA) sense 5'-GCTCTGGTGTGTGACAATGG-3', antisense 5'-CACGATGGATGGGAAAACAG-3'; GAPDH sense 5'-CTCCCTCAAGATTGTCAGCAA-3', antisense 5'-GTCAGATCCACAACGGATACATT-3'. Gene expression was quantitatively analyzed 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., Newton 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). Briefly, lungs were homogenized in 5 ml of 0.5 mol/l acetic acid containing 1 mg pepsin (Sigma Chemical, St Louis, MO, USA) per 10 mg tissue residue. Each sample was incubated for 24 h at 4°C with stirring. After centrifugation, 100 μl of each supernatant was assayed. 1 ml of

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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 mol/l NaOH) included in the kit, and the optical density was evaluated at 540 nm using a spectrophotometer. Values in the test samples were compared to 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 using 12 % SDS-PAGE and electroblotted onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were then incubated with a rabbit anti-collagen type I polyclonal antibody (1:10,000; Abcam), a rabbit anti-α-SMA monoclonal antibody (1:50,000; Epitomics, Burlingame, CA, USA), a rabbit anti-extracellular signal-regulated protein kinase monoclonal antibody (1:2,000; Cell Signaling Technology, MA, USA), or a mouse anti-β-actin monoclonal antibody (1:100,000; Sigma-Aldrich, St. Louis, MO, USA). After incubation with the primary antibody, the membranes were probed with horseradish peroxidase-conjugated secondary antibody (1:20,000; anti-mouse, Pierce, Rockford, IL, USA). 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^{o}C for light microscopic evaluation.

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The sampled tissues were dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Seven μm tissue sections were stained with hematoxylin and eosin (H&E), and examined by a pathologist who was blinded to the protocol and experimental groups. Five random high power (×400) fields were evaluated by the presence of hemorrhage, alveolar septal thickness, and inflammatory cell infiltration, using a scale of 0-4. 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 using a 10× objective lens and a Nikon Eclipse E600 microscope. A minimum of four randomly selected fields were captured at ×100 magnification for each section using a digital camera and imported into a computerized image analysis system (Image-Pro Plus 5.1 for Windows, Media Cybernetics, Silver Spring, MD, USA). The automatic object density process was used for 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 using one-way analysis of variance (ANOVA) with post-hoc Scheffe's test. Survival rate was evaluated using the Kaplan-Meier method and the log-rank test was used for comparisons between treatment groups. Differences were considered significant at $P < 0.05$.

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Results

Body Weight, Lung Weight, and the 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 seven 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 when compared with room-air controls on postnatal days seven 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 seven and significantly larger in hyperoxia-exposed rats when 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 seven and 21.

Survival. Hyperoxia decreased survival rate, mainly on postnatal day 7, reaching 70% in the hyperoxia + vehicle group (Figure 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

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showed normal lung structure on postnatal day seven, and there was no evidence of tissue injury (Fig. 3A, a and b). On postnatal day seven, the lungs of the hyperoxia-exposed rats contained larger, more thin-walled air spaces when compared with room air-exposed rats (Fig. 3A, c and d; Table 3). The changes in the histologic scores in the categories of hemorrhage and interstitial inflammation were not statistically significant on postnatal day seven (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 to room air-exposed rats and losartan treatment decreased macrophage number on postnatal days seven and 21 (Table 3 and 4).

Effects of Hyperoxia on Collagen I and α-SMA mRNA Expression. Hyperoxic exposure for seven and 21 days significantly increased collagen type I and α-SMA mRNA expression compared to room air-exposed rats (Fig. 4, A and B). The increased collagen type I and α-SMA mRNA expression significantly diminished after losartan treatment on postnatal days seven and 21. This study only determined collagen type I expression, and not other collagen types, because it constitutes > 65 % of the total lung collagen in normal lungs (Cairns and Walls, 1997).

Total Collagen and Collagen Density. Hyperoxia treatment of newborn rats significantly increased total lung collagen compared to room air-exposed rats on postnatal days seven and

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21 (Fig. 5, A and B). Losartan treatment inhibited hyperoxia-induced increase of total collagen on postnatal days seven and 21. Masson's trichrome staining further verified the presence of pulmonary fibrosis (Fig. 6). Hyperoxia-exposed rats had widespread collagen deposition in both peribronchial and parenchymal portions of the lung. In contrast, the collagen deposition in hyperoxia-exposed and losartan-treated rats was significantly attenuated.

Effects of Hyperoxia on α-SMA, Collagen I, and ERK Protein Expression. α-SMA is a widely used marker for myofibroblast differentiation. Compared with control pups, hyperoxia-treated pups demonstrated significantly increased α-SMA protein expression on postnatal days seven and 21 (Fig. 7, A and B). This indicated the presence of hyperoxia-induced pulmonary fibrosis in the newborn rats. Hyperoxic exposure for seven days significantly increased collagen type I and ERK 1/2 protein expression compared to room air-exposed rats (Fig. 7, A and B). A further increase in hyperoxia-induced collagen I and ERK 1/2 protein expression occurred on postnatal day 21. The augmented α-SMA, p-ERK, and collagen type I protein expressions significantly diminished after losartan treatment on postnatal days seven and 21.

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 seven and 21. The significant decreases in body weight and lung weight in hyperoxic animals on postnatal days seven and

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21 (Tables 1 and 2) and the histological evidence of lung damage on postnatal day seven (Fig. 2) were consistent with previous studies showing acute lung injury in newborn rats exposed to prolonged hyperoxia (Couroucli et al., 2006; Chen et al., 2007). This study tested the hypothesis that an AT_1R antagonist would attenuate hyperoxia-induced lung fibrosis in newborn rats. The main finding of this study is that the AT_1R antagonist losartan attenuated hyperoxia-induced lung fibrosis. This protection of lung fibrosis by losartan treatment (Figs. 3-4) was consistent with the hypothesis that losartan protects newborn rats from lung fibrosis induced by oxygen. These results suggest that AT_1R plays a significant role in the pathogenesis of hyperoxia-induced lung fibrosis.

AT_1R 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 AT_1R antagonists might act on the lung include decreased vascular tone, decreased vascular permeability, and altered fibroblast activity (Marshall, 2003). Thus, it is probable that anti-fibrotic 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 four, and

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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 three 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 seven and 21, respectively (Tables 1 and 2). The lung weight of hyperoxia-exposed rats also significantly decreased when compared to 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 the 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 seven 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 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

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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 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 (Tomasek et al., 2002). This study identified elevated collagen type I and α-SMA expressions following 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. Following Masson's trichrome staining, collagen presented as a dense bluish-tinged material, as shown surrounding the small and large airways and perivascular interstitium in Figure 4. Increased collagen deposition in the interstitum and inter-alveolar septum occurred in hyperoxia-exposed rats when compared to room air-exposed rats on postnatal days seven and 21 (Fig. 4, A and B). In contrast, hyperoxia-exposed and losartan-treated rats exhibited significantly less collagen deposition compared to room air- and hyperoxia-exposed and vehicle-treated rats.

This study demonstrated that hyperoxia increased p-ERK expression (Fig. 5).

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Mitogen-activated protein kinases (MAPKs) act as signal transducers in response to cellular stresses. A prior investigation linked ERK and p38 MAPK to AT_1R activation (Li et al., 2005). The group's previous study demonstrated that ERK 1/2, 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 ERK 1/2 subsequent to hyperoxic exposure on postnatal days seven (a ~two-fold increase) and 21 (a ~four-fold increase). The AT_1R antagonist losartan decreased the hyperoxia-induced ERK 1/2 protein expression. These 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_1R antagonists in protecting against hyperoxia-induced lung fibrosis. This study also reveals that an AT_1R 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_1R antagonist therapy may provide a novel strategy to prevent hyperoxia-induced lung fibrosis.

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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.

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Footnotes

This work was supported by a grant from the National Science Council, Taiwan [NSC 97-2314-B-038-014].

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Figure Legends

Fig. 1. Experimental design of the study.

Fig. 2. Effects of losartan on survival rate in room air- and hyperoxia-exposed rats. Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Kaplan-Meier curve representation of survival of newborn rats exposed to room air or hyperoxia (circles and rectangles, respectively), and either treated with vehicle (open symbols) or receiving losartan (closed symbols). a denotes significant differences from room air-exposed group curve at $P < 0.01$.

Fig. 3. Morphology of representative lung sections from room air- and hyperoxia-exposed rats (H&E, ×100). Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day seven (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; and d, hyperoxia-exposed rats treated with losartan.

Fig. 4. Effects of losartan on collagen I and α-SMA mRNA expression in room air- and hyperoxia-exposed rats. Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day seven (A) or 21 (B). Collagen I and α-SMA mRNA expression were analyzed using real-time PCR. Values represent means ± S.D. ($n = 3$). One-way ANOVA, followed by

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post-hoc Scheffe's test, were used to evaluate the statistical significance of differences between individual groups. a denotes significant differences from room air + vehicle at $P < 0.05$. b denotes significant differences from room air + vehicle, room air + losartan, and hyperoxia + losartan at $P < 0.01$. c denotes significant differences from room air + vehicle, room air + losartan, and hyperoxia + losartan at $P < 0.05$.

Fig. 5. Effects of losartan on total collagen contents in room air- and hyperoxia-exposed rats. Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day seven (A) or 21 (B). Total collagen contents were measured by the Sircol Collagen Assay. Values represent means ± S.D. ($n = 4$). One-way ANOVA, followed by post-hoc Scheffe's test, were used to evaluate the statistical significance of differences between individual groups. a denotes significant differences from room air + vehicle, room air + losartan, and hyperoxia + losartan at $P < 0.001$.

Fig. 6. Effects of losartan on collagen density in room air- and hyperoxia-exposed rats. Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day seven (A) or 21 (B). Lung tissues were stained with Masson's trichrome and collagen densities were quantified using a computerized image analysis system (100× magnification). Values represent means ± S.D. ($n = 4$). One-way ANOVA, followed by post-hoc Scheffe's test, were

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used to evaluate the statistical significance of differences between individual groups. a denotes significant differences from room air + vehicle, room air + losartan, and hyperoxia + losartan at $P < 0.001$. b denotes significant differences from room air + vehicle, room air + losartan, and hyperoxia + losartan at $P < 0.01$.

Fig. 7. Effects of losartan on α-SMA, collagen I, and ERK protein expression in room air- and hyperoxia-exposed rats. Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal days seven or 21. α-SMA, collagen I, and ERK protein expression were analyzed using Western blot. (A), the densitometry unit of protein expression in room air-exposed lungs was assigned as 1 after being normalized to β-actin (B). Values represent means ± S.D. (n = 4). One-way ANOVA, followed by post-hoc Scheffe's test, were used to evaluate the statistical significance of differences between individual groups. a denotes significant differences from room air + vehicle, room air + losartan, and hyperoxia + losartan at $P <$ 0.001.

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TABLE 1

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

Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day seven. Values represent means ± S.D.

Treatment	*n*	Body weight (g)	Lung weight (g)	Lung/body weight (%)
Room air + vehicle	6	11.61 ± 0.84	0.22 ± 0.03	1.87 ± 0.25
Room air + losartan	7	11.79 ± 0.89	0.24 ± 0.04	2.01 ± 0.25
Hyperoxia + vehicle	8	9.13 ± 0.84[a]	0.16 ± 0.04[b]	1.77 ± 0.34
Hyperoxia + losartan	8	9.30 ± 0.41[a]	0.15 ± 0.02[a]	1.60 ± 0.17[c]

[a] Different from room air-exposed rats at $P < 0.001$.

[b] Different from room air-exposed rats at $P < 0.01$.

[c] Different from room air + losartan group at $P < 0.05$.

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TABLE 2

Body weight, lung weight, and the lung/body weight ratio in room air- and hyperoxia-exposed rats on postnatal day 21.

Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day 21. Values represent means ± S.D.

Treatment	*n*	Body weight (g)	Lung weight (g)	Lung/body weight (%)
Room air + vehicle	6	44.28 ± 6.68	0.39 ± 0.05	0.89 ± 0.05
Room air + losartan	6	44.55 ± 5.89	0.40 ± 0.03	0.90 ± 0.06
Hyperoxia + vehicle	6	14.36 ± 1.53[a]	0.21 ± 0.02[a]	1.45 ± 0.10[a]
Hyperoxia + losartan	8	15.62 ± 1.34[a]	0.23 ± 0.04[a]	1.48 ± 0.14[a]

[a] Different from room air-exposed rats at $P < 0.001$.

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TABLE 3

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

Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day seven. Values represent means ± S.D.

Treatment	*n*	Hemorrhage	Alveolar septal thickness	Interstitial inflammation	Macrophage /lung field
Room air + vehicle	6	0.0 ± 0.0	1.5 ± 0.5	0.0 ± 0.0	7.3 ± 1.3
Room air + losartan	7	0.1 ± 0.2	1.3 ± 0.2	0.0 ± 0.0	6.9 ± 1.4
Hyperoxia + vehicle	8	0.1 ± 0.1	0.0 ± 0.1[a]	0.0 ± 0.1	20.8 ± 4.3[b]
Hyperoxia + losartan	8	0.0 ± 0.0	0.6 ± 0.3[a]	0.0 ± 0.0	6.0 ± 1.4

[a] Different from room air-exposed rats at $P < 0.001$.

[b] Different from room air-exposed rats and hyperoxia + losartan group at $P < 0.001$.

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TABLE 4

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

Newborn rats were exposed to room air or hyperoxia and treated with vehicle or losartan as described in *Materials and Methods*. Animals were sacrificed on postnatal day 21. Values represent means ± S.D.

Treatment	*n*	Hemorrhage	Alveolar septal thickness	Interstitial inflammation	Macrophage /lung field
Room air + vehicle	6	0.0 ± 0.0	1.0 ± 0.2	0.0 ± 0.0	8.5 ± 1.0
Room air + losartan	6	0.0 ± 0.0	1.2 ± 0.2	0.0 ± 0.0	8.0 ± 1.8
Hyperoxia + vehicle	6	0.3 ± 0.3[a]	1.0 ± 0.4	0.2 ± 0.3	13.8 ± 2.3[b]
Hyperoxia + losartan	8	0.0 ± 0.0	1.0 ± 0.1	0.0 ± 0.0	8.8 ± 2.0

[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 $P < 0.01$.

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Fig. 1.

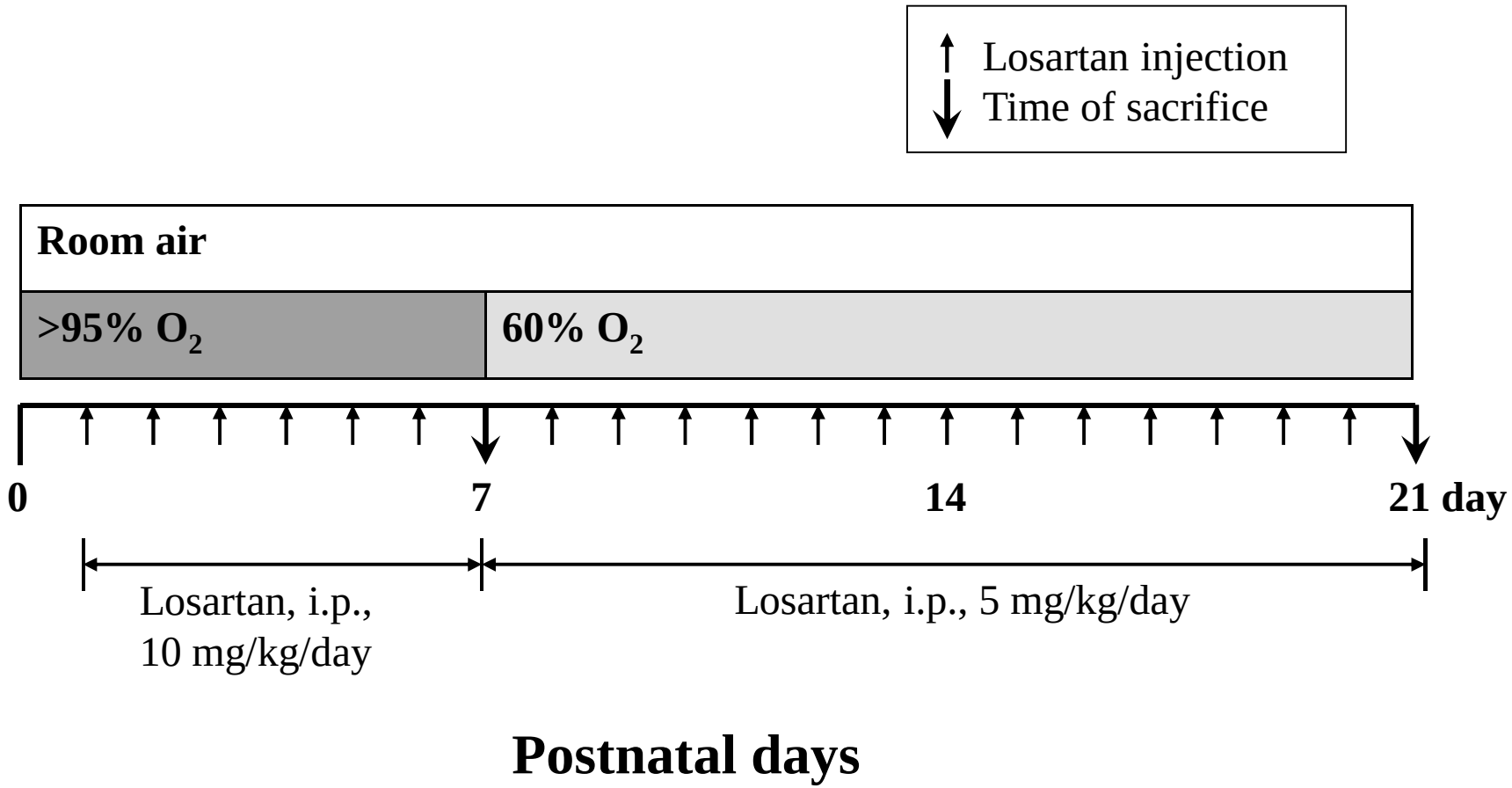

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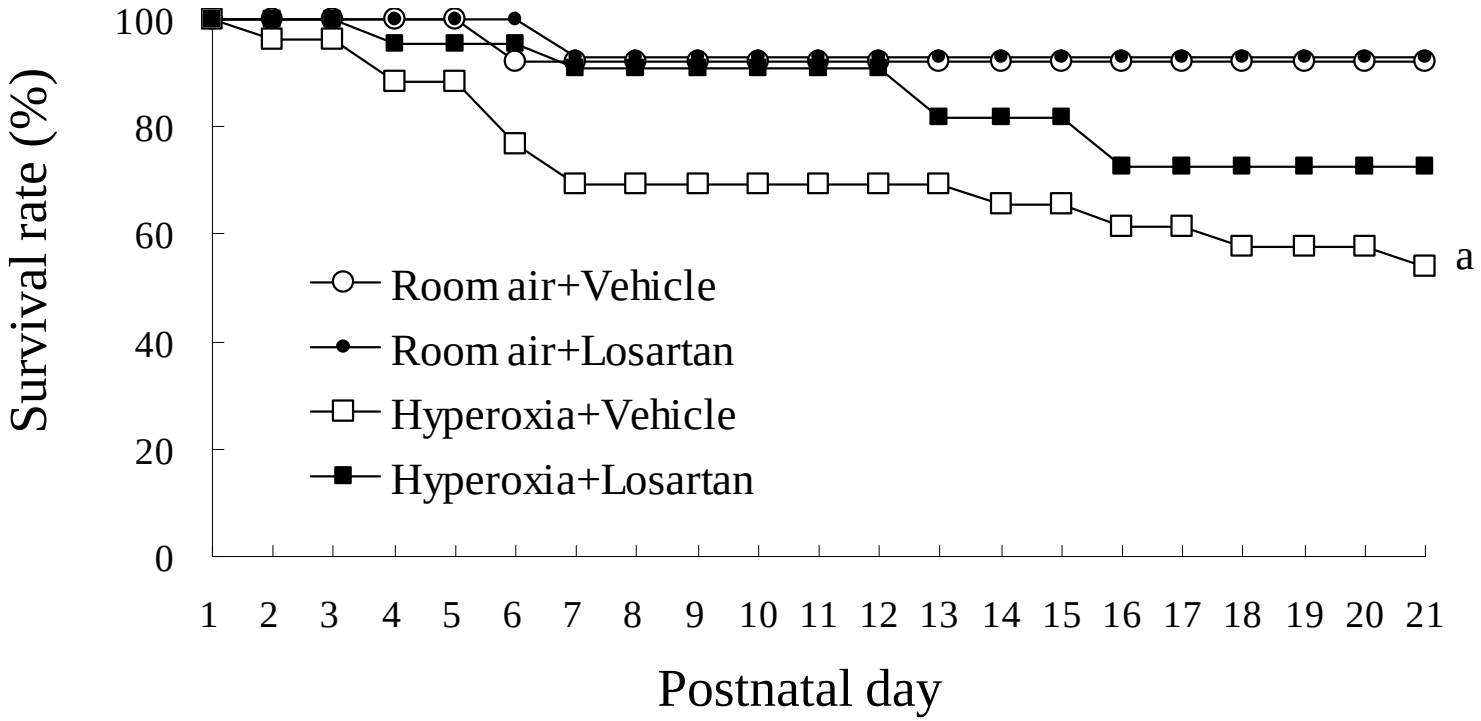
Fig. 2.



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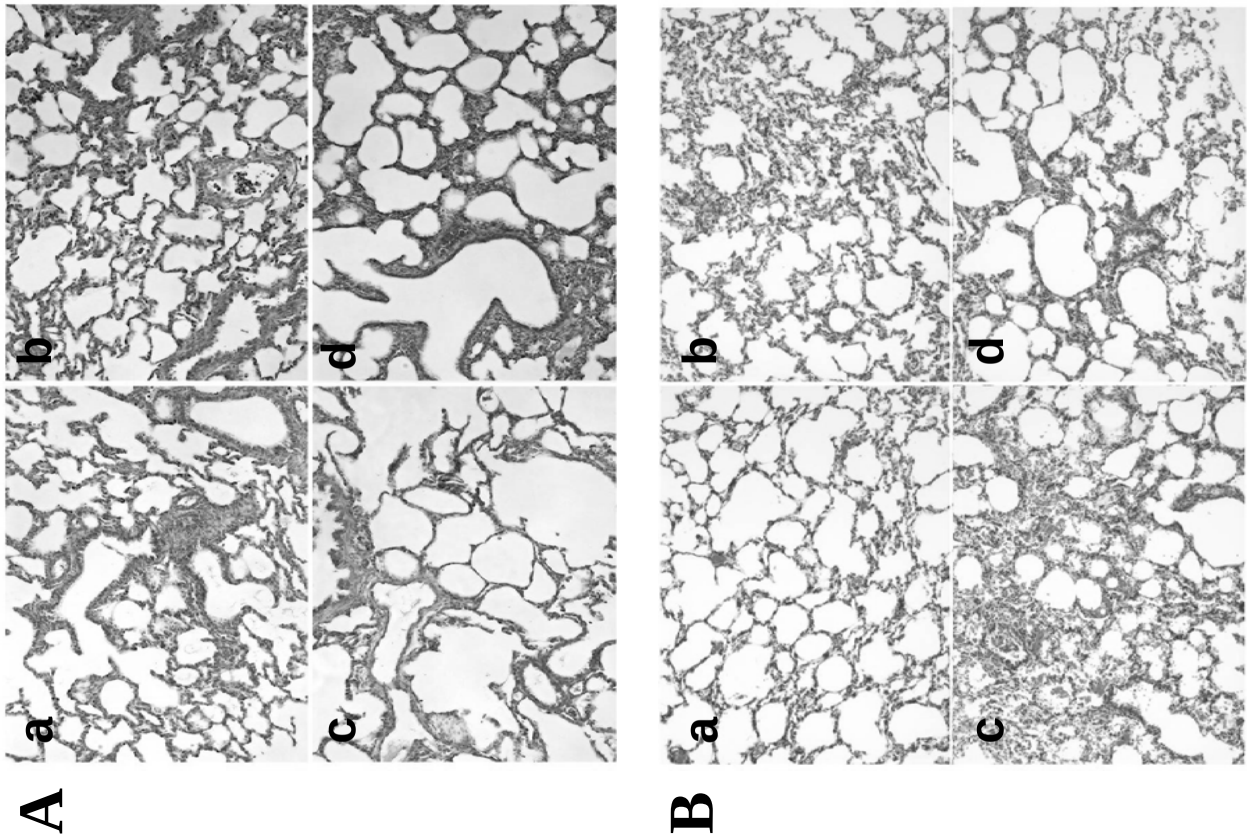

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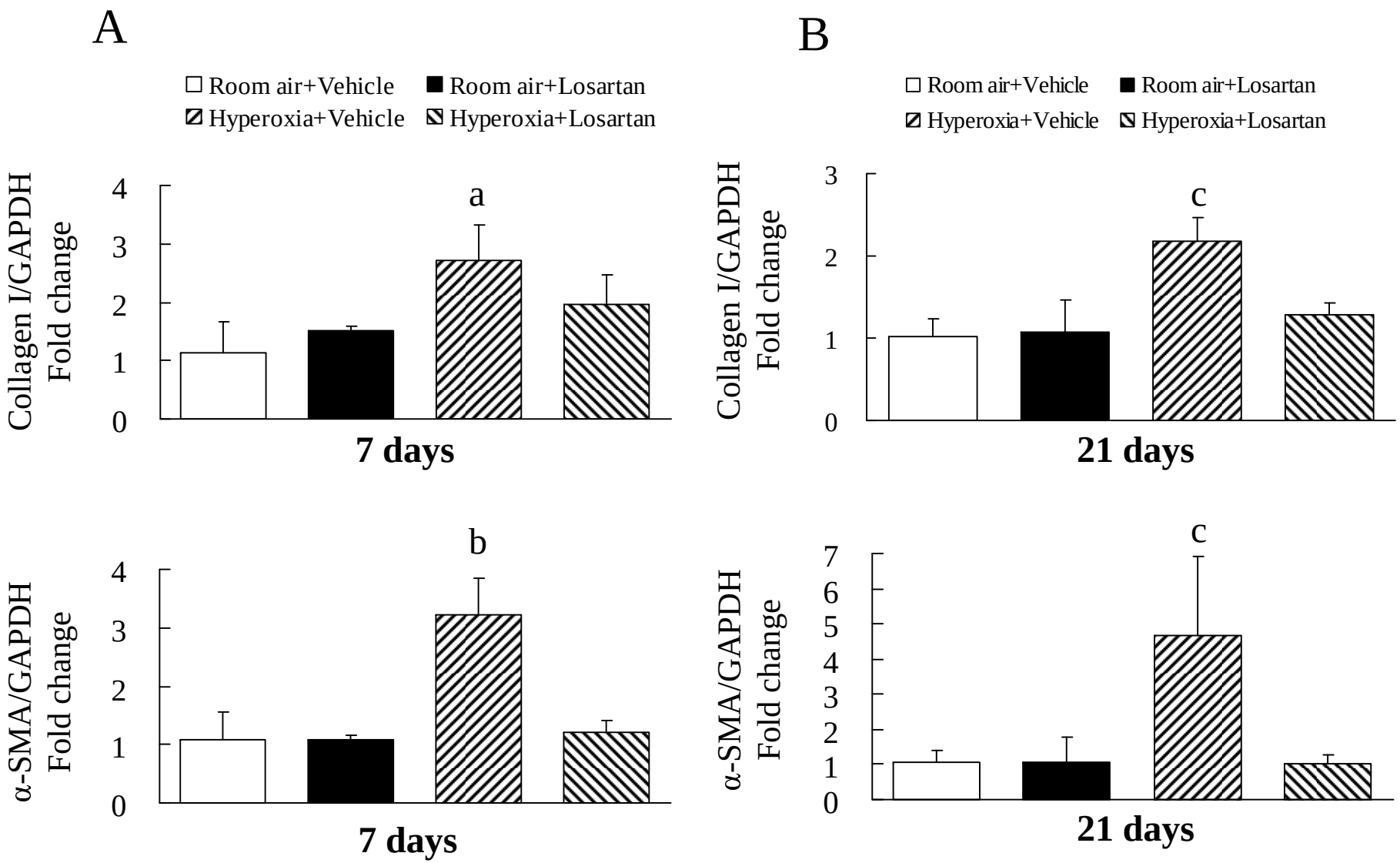

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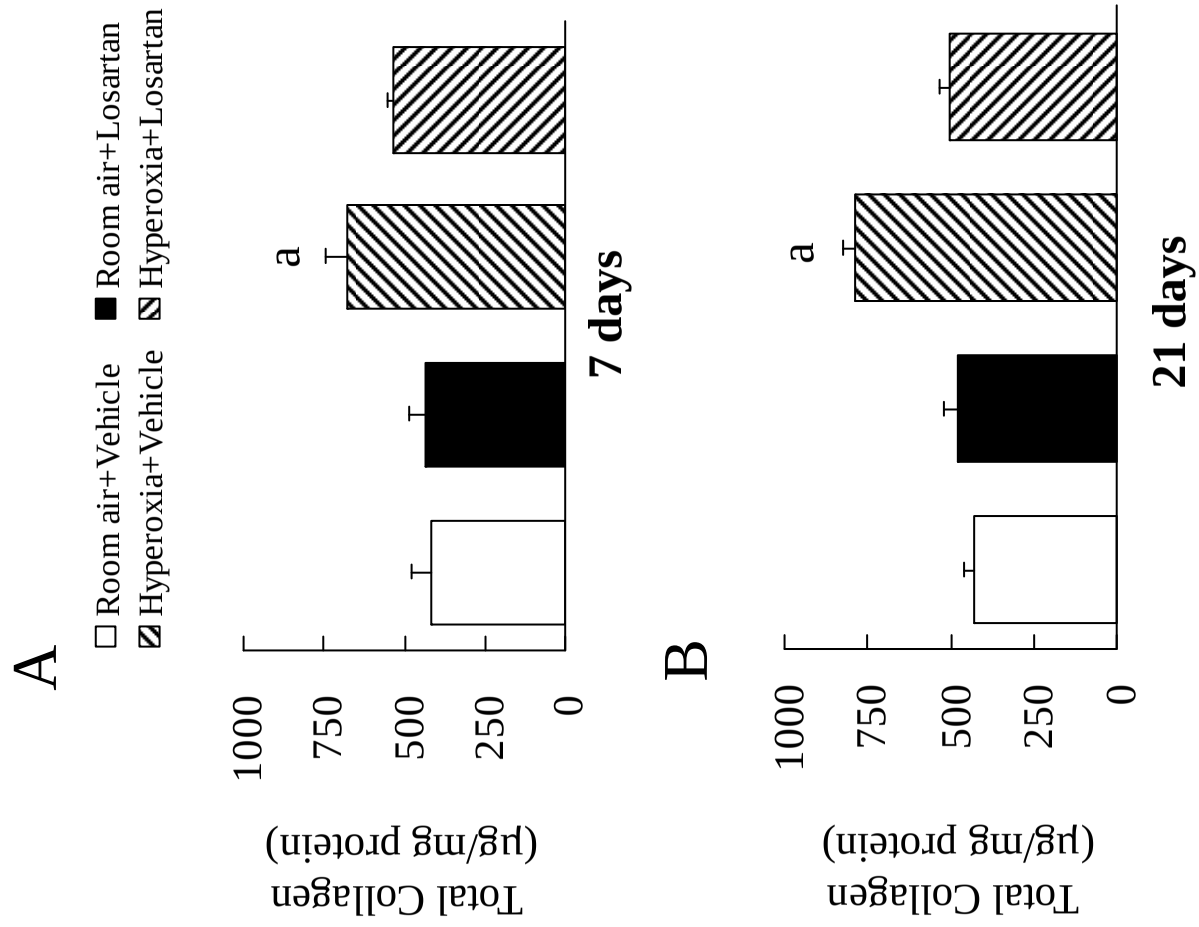
Fig. 5.



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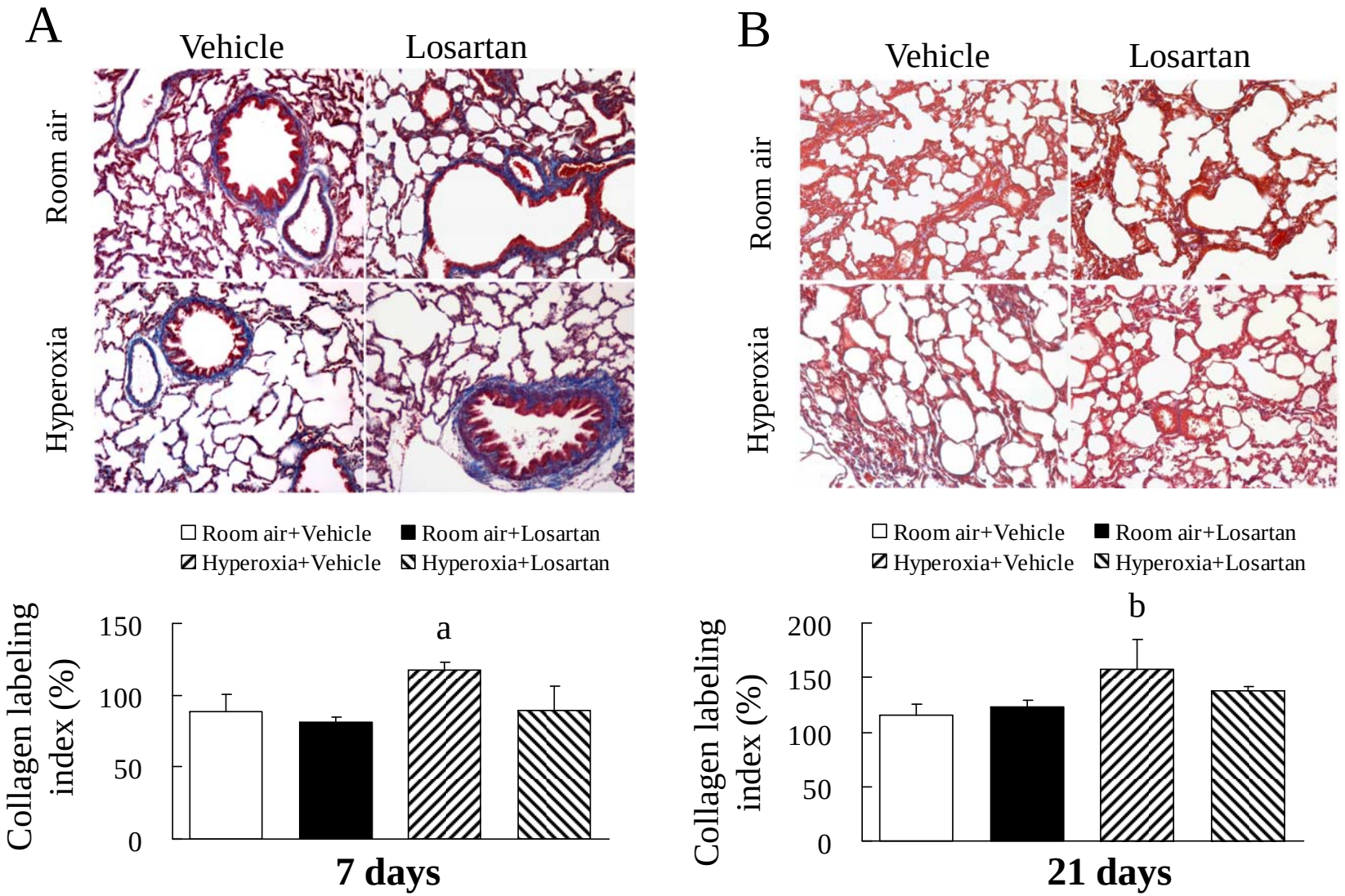

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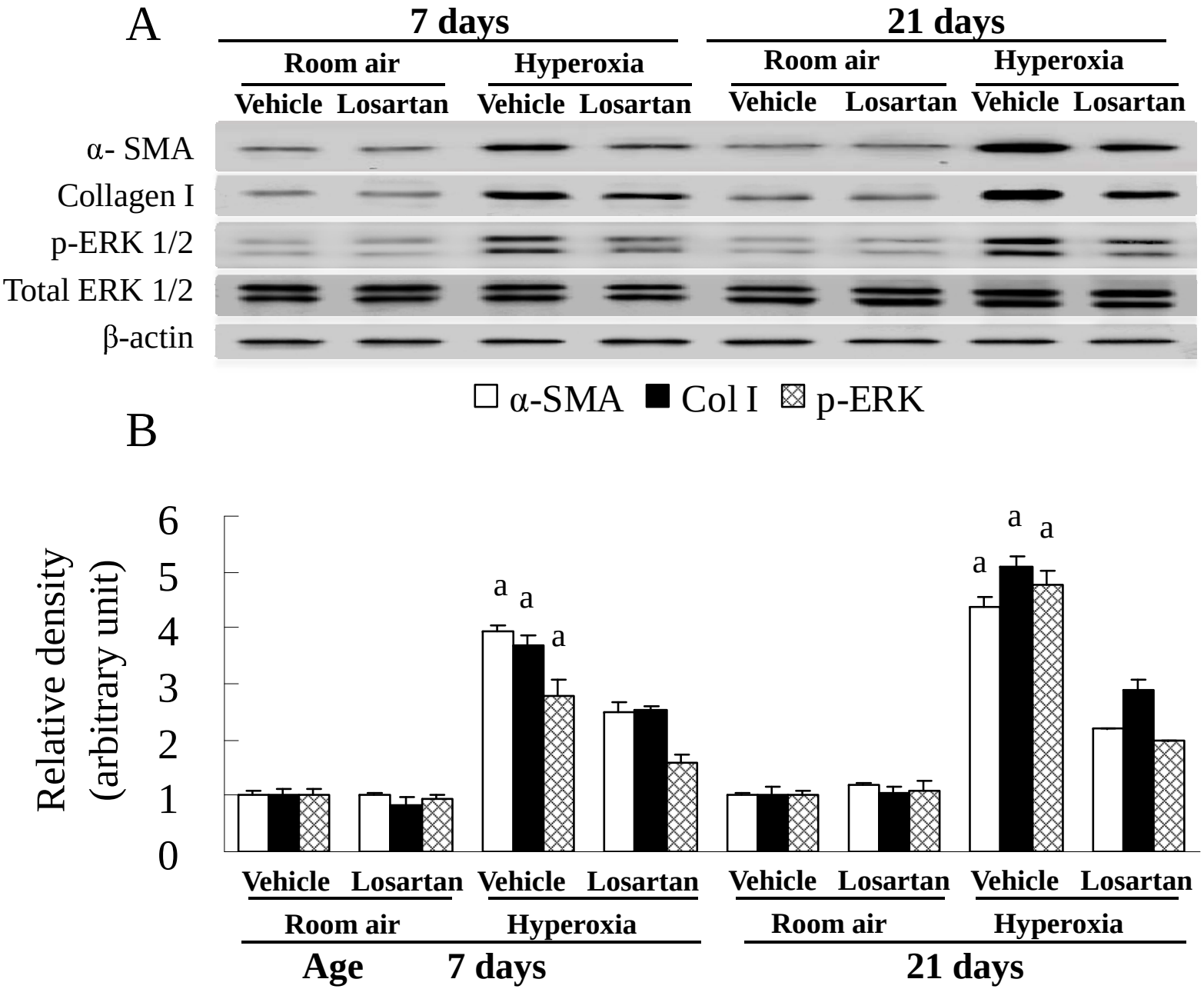
Fig. 7.



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