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Original Article

Rho-kinase inhibitor Y-27632 attenuates pulmonary hypertension in hyperoxia-exposed newborn rats

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Aim: To test the hypothesis that neonatal hyperoxia induced pulmonary hypertension accompanied by increased Rho-kinase expression in rat lungs and that Rho-kinase inhibitor could attenuate right ventricular hypertrophy and pulmonary arterial remodeling.
Methods: Newborn rats were exposed to >95% O_2 in the first week after birth, then to 60% O_2 in the following 2 weeks. Control pups were exposed to room air over the same periods. The pups were injected with either Rho-kinase inhibitor Y-27632 (10 mg·kg^{-1}·d^{-1}, ip) or vehicle from postnatal d 14 to 20. Lung and heart tissues were collected on postnatal d 7 and 21. Rho-kinase activity in lungs was measured using Western blotting and immunohistochemistry. The right ventricular hypertrophy and arterial medial wall thickness (MWT) were assessed morphologically.
Results: Rho-kinase activity in lungs was comparable between the hyperoxic and control pups on postnatal d 7, but it had a more than 2-fold increase in the hyperoxic pups on postnatal d 21. Moreover, the hyperoxic exposure induced structural features of pulmonary hypertension, as shown by the right ventricular hypertrophy and significantly increased arterial MWT. Administration with Y-27632 effectively blocked the hyperoxia-induced increase of Rho-kinase activity in lungs, and attenuated the right ventricular hypertrophy.
Conclusion: Rho-kinase inhibitor may be a novel therapy for attenuating the hyperoxia-induced structural changes in pulmonary hypertension.

Keywords: neonate; hyperoxia; lung injury; pulmonary hypertension; Rho-kinase; Y-27632

Introduction

Oxygen therapy is an important component in the management of various conditions of respiratory distress in neonates. However, prolonged exposure to hyperoxia in neonates can cause hyperoxic lung injury. Neonatal mice exposed to hyperoxia showed diffuse alveolar damage, increased terminal air space size, and increased lung fibrosis that was similar to human bronchopulmonary dysplasia[1, 2]. Rat pups exposed to high oxygen levels during the alveolar period exhibit decreased alveolarization and pulmonary hypertension, as evidenced by increased muscularization of the peripheral arteries and medial hypertrophy of the muscular arteries[3–7].

The small GTP-binding protein Rho and its downstream effector Rho-kinase play an important role in regulating vascular smooth muscle tone[8]. The Rho-kinase system is constitutively active in regulating vasoconstrictor tone, and upregulation of this pathway occurs in various cardiovascular diseases[9, 10]. Rho-kinase is found to be upregulated in animal models of pulmonary hypertension, and Rho-kinase inhibitors decrease the pulmonary arterial pressure in rodents with monocrotaline and chronic hypoxia-induced pulmonary hypertension[11–13]. Rho-kinase has been identified as a potential therapeutic target in pulmonary hypertension[14]. However, the therapeutic effect of Rho-kinase inhibitor on hyperoxia-induced pulmonary hypertension is unknown. This study tested the hypotheses that neonatal hyperoxia induces pulmonary hypertension and is accompanied by an increase in Rho-kinase expression and that Rho-kinase inhibitor may attenuate heart structural changes in rats.

Materials and methods

Animals and exposure to hyperoxia

The study was performed in accordance with guidelines provided and approved by the Animal Care and Use Committee

of Taipei Medical University (Taiwan, China). Time-dated pregnant Sprague-Dawley rats were housed in individual cages. Within 12 h of birth, litters were pooled and randomly redistributed to the newly delivered mothers, and then exposed to either hyperoxia (experimental group) or room air (control group). Nursing mothers were rotated between the experimental and control litters every 24 h to avoid oxygen toxicity in the mothers. The control groups were kept in normoxia for 1 and 3 weeks. The hyperoxia groups were exposed to >95% O_2 for 1 week and were then placed in an environment with 60% O_2 for a further 2 weeks. Oxygen exposure was carried out in a modified controller for the basic model (NexBiOxy, Hsinchu, Taiwan, China). Rat pups were injected intraperitoneally with either Rho-kinase inhibitor Y-27632 (Sigma-Aldrich, St Louis, MO, USA) at a dose of 10 mg·kg^{-1}·d^{-1} or an equivalent volume of vehicle (normal saline) from postnatal d 14 to 20 (Figure 1). The dose and duration of Y-27632 were based on Ziino et al and Xu et al studies[15, 16]. Animals were killed by an intraperitoneal injection of pentobarbital sodium and exsanguinated by aortic transaction on postnatal d 7 and 21. Lung and heart from room air- and hyperoxia-exposed pups were harvested on postnatal d 7 and 21. Body and heart and lung weights were recorded at the time of sacrifice.
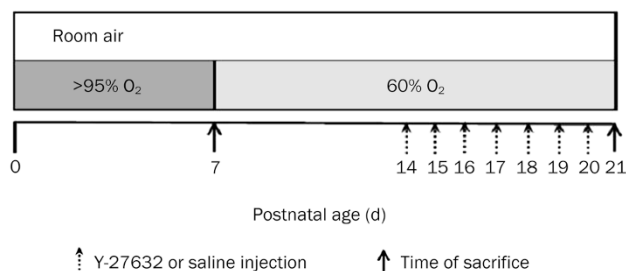

Figure 1. Schematic diagram of the experimental design.

Western blotting analysis for ERM family

To quantify Rho-kinase activity, we performed Western blotting analysis for the total and phosphorylated ERM (ezrin, radixin, and moesin) family, a substrate of Rho-kinase (n=4 samples per group). Activated Rho-kinase has been shown to directly phosphorylate COOH-terminal threonine residues of the ERM proteins to regulate their function, and relative Rho-kinase activity is determined as a measure of the ratio of phosphorylated to total ERM[11]. The primary antibodies used in this study were goat anti-p-ERM (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), goat anti-ERM (Santa Cruz, 1:200), and mouse anti-β-actin (Sigma-Aldrich, 1:200 000). After incubation with the primary antibody, the membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-goat, 1:20 000; Pierce, Rockford, IL, USA). Immune complexes were visualized using enhanced chemiluminescence

detection reagents (Pierce). Densitometric analysis was performed to measure the intensity of Western blotting bands using AIDA software (Advanced Image Data Analyzer; Raytest Izotopenmessgeraete, Straubenhardt, Germany).

Immunohistochemistry

Rho activation was evaluated by analysis of site-specific phosphorylation of Rho-kinase substrate myosin-binding subunit of myosin-associated phosphatase type 1 (MYPT1). Slides were incubated with goat polyclonal anti-p-MYPT1 (Thr 696) antibodies (1:100; Santa Cruz) as the primary antibody. Proliferation of pulmonary artery smooth muscle cells was assessed using anti-proliferating cell nuclear antigen (PCNA) rabbit monoclonal antibody (1:100; Epitomics, Burlingame, CA, USA). The sections were then treated with biotinylated rabbit anti-goat IgG (1:200, Jackson ImmunoResesarch Labotories Inc, PA, USA). Thereafter it was reacted with reagents from an ABC kit (Avidin-Biotin Complex, Vector, CA, USA) according to the manufacturer's recommendations. The optical density values of p-MYPT1 were measured using Image pro plus software version 6.0 (Media Cybernetics, USA) under 200×objective lens of each slice for semiquantitative analysis[17]. Proliferation was assessed by examining arteries (20 to 60 μm in diameter) in 4 randomly chosen fields in each section and expressed as a percentage of the total number of arteries examined[18].

Right ventricular hypertrophy and pulmonary arterial remodeling

The hearts were removed and placed in fixative for 48 h at room temperature. After serial dehydration in alcohol, the tissues were embedded in paraffin. The 7 μm lung sections were stained with hematoxylin and eosin for light microscopy and morphometric analysis. Right ventricular hypertrophy (RVH) was assessed by measuring the thickness ratio of right ventricle (RV)/[left ventricle (LV)+interventricular septum (IVS)][8]. The medial wall thickness (MWT) was measured in vessels with an external diameter of 20 to 65 μm. Wall thickness and external diameter were measured under Image-Pro Plus. Fourteen to twenty-six vessels were measured in each animal. The percent medial thickness of an individual vessel was calculated by the following formula: (medial thickness×2×100)/external diameter[19].

Statistical analysis

Data are expressed as the mean±SD. Comparisons between the room air and experimental groups were made using Student's t-test on postnatal d 7 and one-way ANOVA with post-hoc Tukey's test on postnatal d 21. Differences were considered significant at $P<0.05$.

Results

Three pregnant dams were assigned to both room air and hyperoxia groups for 1 week and four pregnant dams were assigned to Rho-kinase inhibition experiment on postnatal d 21. There was no mortality in the rat pups.

### Body weight, organ weight, and organ/body weight ratio (%) in rats

All rat pups survived during the study period. The effects of hyperoxia on the rats' body weight, organ weight, and organ/body weight ratio (%) are shown in Table 1. Rats exposed to hyperoxia exhibited significantly lower body and organ weights compared with room-air controls on postnatal d 7 and 21. Heart/body weight and lung/body weight ratios were significantly lower in the hyperoxia-exposed rats on postnatal d 7, relative to the controls. However, on postnatal d 21, although the hyperoxia-exposed rats still showed significantly lower body and organ weights compared with room air-exposed rats, the difference in heart/body weight ratio between the hyperoxia-exposed and control groups was not statistically significant.

Rho-kinase activity

Lungs from the control and experimental groups showed similar levels of Rho-kinase activity on postnatal d 7, as indicated by the ratio of phosphorylated ERM to ERM (ezrin, radixin, and moesin) (Figure 2A). In the group exposed to hyperoxia, this ratio was significantly increased by postnatal d 21, indicating increased activation of Rho-kinase (Figure 2B). Rho-kinase inhibitor treatment for 1 week reduced the hyperoxia-induced increase of Rho-kinase activity. We also investigated the effects of hyperoxia on Rho-kinase activity in lung tissues by conducting immunohistochemical analysis of p-MYPT1. The immunoreactivity was localized in the smooth muscle cells of the vessels and was enhanced in the lung tissues of hyperoxia-exposed rats on postnatal d 21 (Figure 3A). Decreased p-MYPT1 immunoreactivity was observed in the control rats. Rho-kinase inhibitor treatment for 1 week decreased the hyperoxia-induced increase of p-MYPT1 immunoreactivity (Figure 3B).

Right ventricular hypertrophy and pulmonary arterial remodeling

As an indicator of RVH, we calculated the thickness ratio of RV/(LV+IVS). We also estimated arterial remodeling by measuring the MWT of the small pulmonary arteries because MWT is a surrogate marker of pulmonary hypertension[20]. RV/(LV+IVS) ratios and MWT were comparable between room air- and hyperoxia-exposed rats on postnatal d 7 (Figures
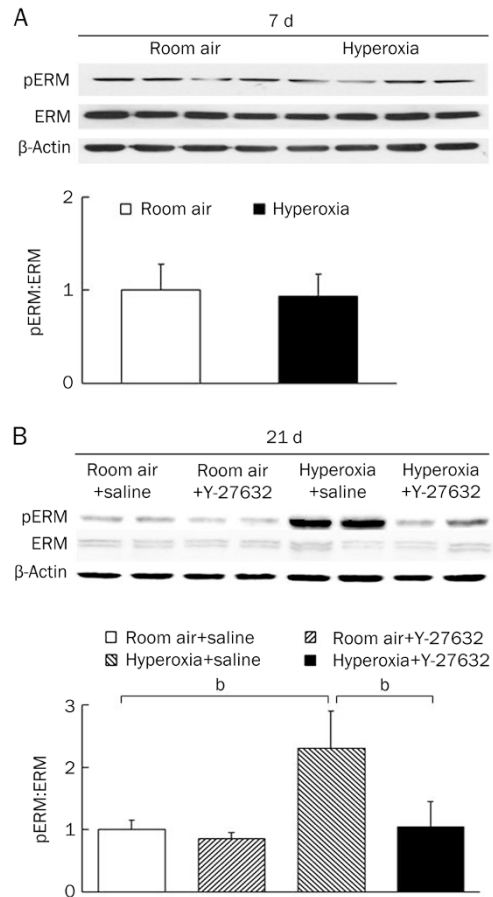

Figure 2. Lung Rho-kinase activity in (A) 7-d-old rats exposed to room air or hyperoxia and (B) 21-day-old rats exposed to room air or hyperoxia from birth and received Y-27632 or saline from postnatal d 14 to 20. Representative Western blotting for phosphorylated (p) ERM proteins and total ERM and scanning densitometry results for quantification of the protein. Rho-kinase activities were comparable between room air- and hyperoxia-exposed rats on postnatal d 7. Hyperoxia+saline group exhibited a significantly higher Rho-kinase activity compared with room air-exposed groups and hyperoxia+Y-27632 groups on postnatal d 21 ($n=4$ samples per group. $^{b}P<0.05$).

4A and 5A). Chronic exposure to hyperoxia was associated with a significant increase in RVH and MWT by 30% and

Table 1. Body weight, organ weight, and organ/body weight ratio in the rats.

Treatment	Age (d)	n	Body weight (g)	Heart weight (g)	Lung weight (g)	Heart/body weight (%)	Lung/body weight (%)
Room air	7	12	14.22±0.99	0.08±0.01	0.29±0.02	0.58±0.04	2.03±0.09
Hyperoxia	7	11	12.44±0.82^{c}	0.07±0.01^{c}	0.20±0.01^{c}	0.53±0.06^{b}	1.57±0.06^{c}
Room air+saline	21	11	36.24±2.24	0.18±0.01	0.36±0.03	0.51±0.04	0.98±0.05
Room air+Y-27632	21	5	34.24±2.81	0.18±0.01	0.34±0.02	0.52±0.03	1.00±0.06
Hyperoxia+saline	21	10	21.08±1.74^{c}	0.12±0.01^{c}	0.25±0.02^{c}	0.56±0.05	1.21±0.06^{c}
Hyperoxia+Y-27632	21	10	21.49±1.40^{c}	0.11±0.01^{c}	0.28±0.02^{c}	0.53±0.04	1.31±0.12^{c}

Values indicate mean±SD. $^{b}P<0.05$, $^{c}P<0.01$, compared with room air or room air+saline/Y-27632 rats at each time point.
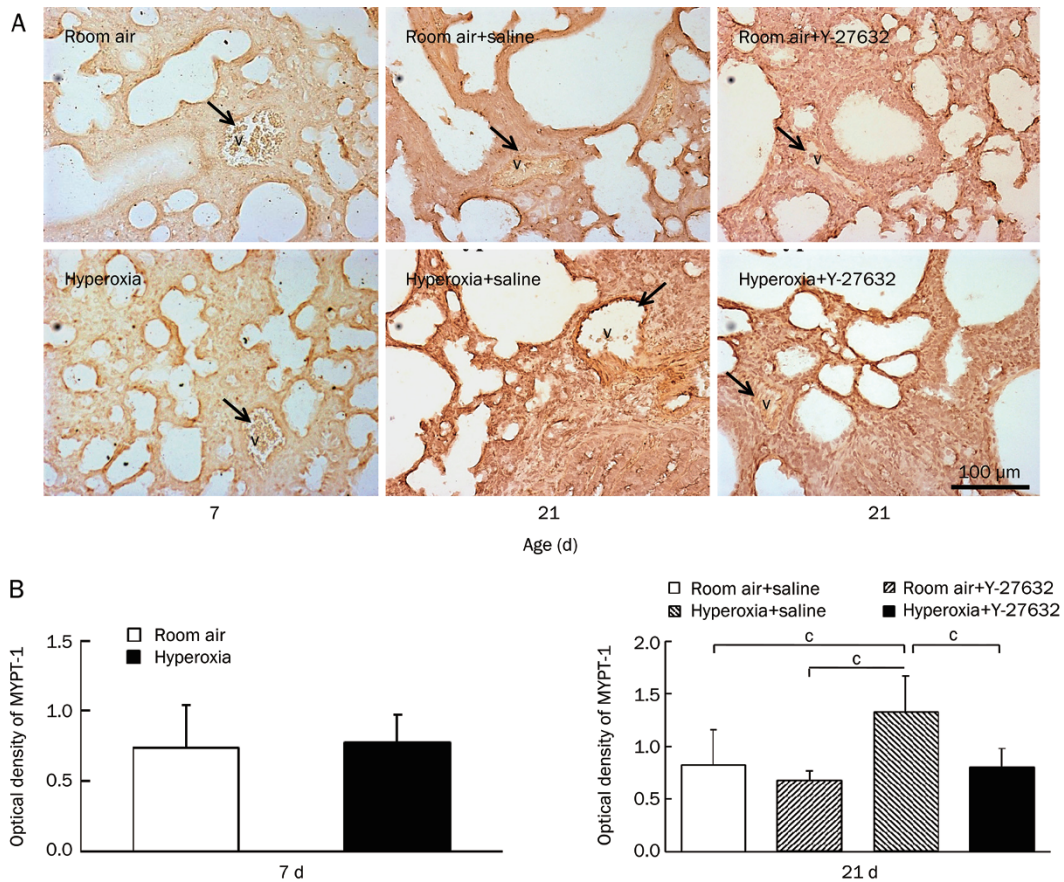
Figure 3. Representative photomicrographs and optical density of p-MYPT1. (A) The ROCK activity was investigated in the rat lung sections by immunostain for p-MYPT1, as described in the Methods section. Positive immunoreactivity is indicated by brown staining. The immunoreactivity was localized in the smooth muscle cells of the vessels (v; arrow) and was enhanced in the lung tissues of hyperoxia-exposed rats on postnatal d 21. Rho-kinase inhibitor treatment for 1 week decreased the hyperoxia-induced increase of MYPT1 immunoreactivity. (B) Optical densities of p-MYPT1 were comparable between room air- and hyperoxia-exposed rats on postnatal d 7. Rats exposed to hyperoxia+saline displayed significantly higher optical density of p-MYPT1 compared with room air-exposed groups on postnatal d 21 ($^cP<0.01$). Rho-kinase inhibitor treatment decreased the hyperoxia-induced increase of p-MYPT1 ($^cP<0.01$ *vs* hyperoxia+saline group).
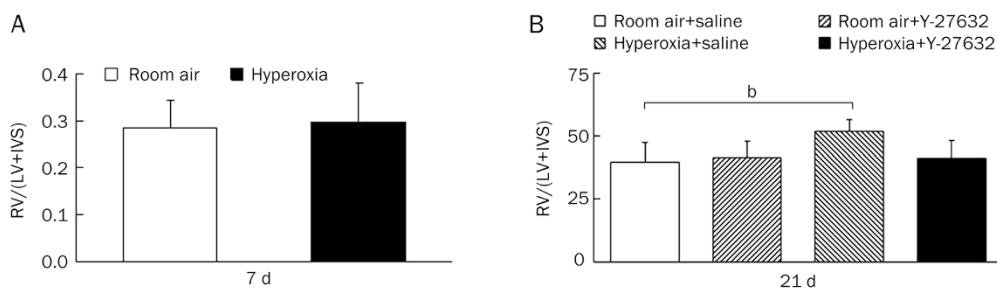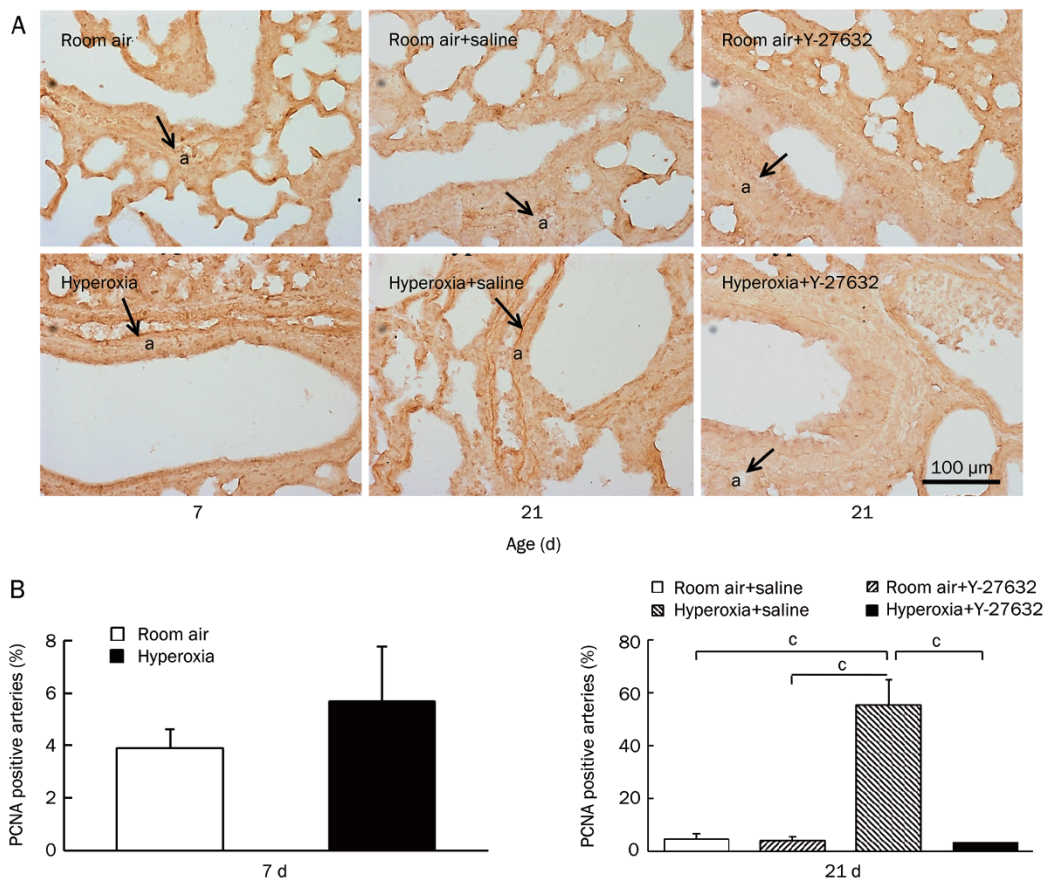

Figure 4. RV/(LV+IVS) ratios in 7-d-old rats exposed to room air or hyperoxia and 21-d-old rats exposed to room air or hyperoxia from birth and received Y-27632 or saline from postnatal d 14 to 20. (A) RV/LV+IVS ratios were comparable between room air- and hyperoxia-exposed rats on postnatal d 7. (B) Rats exposed to hyperoxia+saline displayed significantly higher RVH, as shown by an increased RV/LV+IVS ratio compared with room air+saline group on postnatal d 21 ($^bP<0.05$). Rho-kinase inhibitor treatment for 1 week (hyperoxia+Y-27632 group) tended to decrease the hyperoxia-induced increase of RVH ($P=0.064$ *vs* hyperoxia+saline group).

10% compared to the room air-exposed controls on postnatal d 21, respectively (Figures 4B and 5B). Rho-kinase inhibitor treatment for 1 week (hyperoxia+Y-27632 group) tended to decrease the hyperoxia-induced increase of RVH compared to hyperoxia+saline group on postnatal d 21 ($P=0.064$).
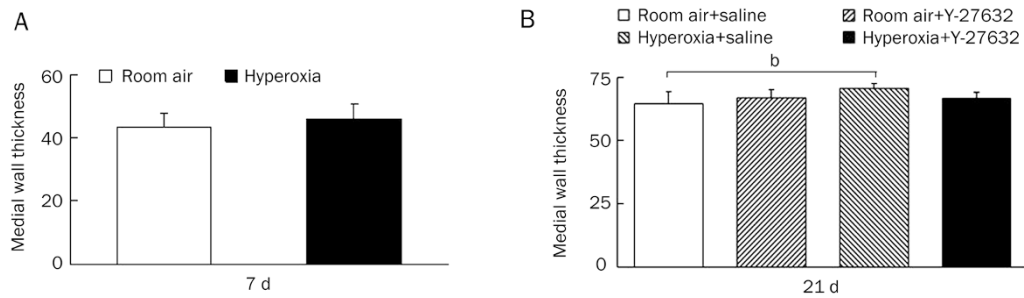
Figure 5. MWT in 7-d-old rats exposed to room air or hyperoxia and 21-d-old rats exposed to room air or hyperoxia from birth and received Y-27632 or saline from postnatal d 14 to 20. (A) MWT values were comparable between room air- and hyperoxia-exposed rats on postnatal d 7. (B) Rats exposed to hyperoxia+saline displayed a significantly higher MWT compared with room air+saline group on postnatal d 21 ([b]$P<0.05$).

Figure 6. (A) Representative PCNA staining and (B) quantification of the percentage of PCNA-positive pulmonary arteries in 7-d-old rats exposed to room air or hyperoxia and 21-d-old rats exposed to room air or hyperoxia from birth and received Y-27632 or saline from postnatal d 14 to 20. PCNA positive cells were found in the smooth muscle layer of small pulmonary artery (a, arrow). The numbers of PCNA-positive pulmonary arteries were comparable between room air- and hyperoxia-exposed rats on postnatal d 7. Rats exposed to hyperoxia+saline had significantly higher numbers of PCNA-positive pulmonary arteries compared with room air-exposed groups on postnatal d 21 ([c]$P<0.01$). Rho-kinase inhibitor treatment decreased the hyperoxia-induced increase of PCNA-positive pulmonary arteries ([c]$P<0.01$ *vs* hyperoxia+saline group). PCNA, proliferating cell nuclear antigen.

Pulmonary artery cell proliferation

Representative PCNA staining and quantification of the percentage of PCNA-positive pulmonary arteries are presented in Figure 6. The number of PCNA-positive pulmonary arteries was comparable between room air- and hyperoxia-exposed rats on postnatal d 7. Rats exposed to hyperoxia+saline had significantly higher numbers of PCNA-positive pulmonary arteries compared with room air-exposed groups on postnatal d 21. Rho-kinase inhibitor treatment decreased the hyperoxia-induced increase of PCNA-positive pulmonary arteries.

Discussion

Our *in vivo* model showed that exposure of neonatal rats to hyperoxia and further prolonged exposure to a lower concentration of oxygen induced pulmonary hypertension, as shown by increased RVH and arterial MWT. These phenomena were associated with increased p-ERM expression, and Rho-kinase inhibitor treatment reduced Rho-kinase activity and tended to decrease the hyperoxia-induced increase in RVH in the third postnatal week. These results suggested that Rho-kinase inhibitor may provide a novel therapy in attenuating hyperoxia-induced structural changes in pulmonary hypertension.

Murine alveolar development begins on postnatal d 4 and saccular division is completed by the fourteenth day[21]. Newborn rats are appropriate for the study of neonatal oxygen injury because the developmental stage of the rodent lung at birth parallels that of the human preterm neonate at 24 to 28 weeks of gestation[3]. During the first 3 weeks of this study, the body weight of experimental rats was approximately 85% and 60% that of the controls on postnatal d 7 and 21, respectively; lung and heart weights were affected similarly to body weight. The hyperoxia-exposed rats exhibited significantly lower body and organ weights and organ/body weight ratios compared with room-air controls on postnatal d 7. However, on postnatal d 21, although the hyperoxia-exposed rats still showed significantly lower body and organ weights, the difference in heart/body weight ratio between the hyperoxia-exposed and control groups was not statistically significant. Rho-kinase inhibitor Y-27632 treatment for 1 week does not influence body weight, organ weight, or organ/body weight ratio in room air- or hyperoxia-exposed rats on postnatal d 21.

The small GTPase RhoA (a member of the Rho family of small GTP-binding proteins) and its downstream effector Rho-kinase play a major role in regulating various cellular functions[22, 23]. Rho/Rho kinase-mediated Ca^{2+} sensitization is a key component in the sustained vasoconstriction induced by G protein-coupled receptor agonists. Y-27632 is the best-characterized Rho kinase inhibitor that selectively targets P160-Rho kinase from the family of Rho-associated protein kinases[24]. Although Y-27632 has been shown to attenuate the hypoxic pulmonary vasoconstrictor response in rodents, the effects of Y-27632 on pulmonary arterial remodeling induced by hyperoxia have not been examined in the intact rats[25]. Enomoto *et al* found that short-term hyperoxia exposure immediately after birth increased Rho-kinase activity in male newborn rat lungs[26]. In this study, we measured whole lung Rho-kinase activity in both sexes and found that Rho-kinase inhibitor treatment for 1 week decreased the hyperoxia induced increase of Rho-kinase activity on postnatal d 21. Rho-kinase inhibitor treatment decreased RVH and pulmonary arterial remodeling in hyperoxia-exposed rats. However, the differences did not reach statistical significance. The discrepancy between ours and Enomoto *et al* studies may be due to different duration of hyperoxia exposure and age of study.

An increasing number of studies implicate oxidant stress as an important initiating factor in pulmonary hypertension[27]. Oxidant stress and the consequent upregulation of G protein-coupled receptor ligands that are critical to the pathogenesis of pulmonary hypertension are known to activate RhoA[28]. Recent studies have implicated the small GTPase, RhoA and its effector protein Rho-kinase, as a key pathway regulating pulmonary vascular tone[29]. We speculate that these reactive oxygen species-induced mediators lead to activation of the RhoA/ROCK pathway in our rat model of pulmonary hypertension.

In summary, we found that exposing neonatal rats to 3-week hyperoxia caused pulmonary hypertension, as shown by increased RVH and arterial MWT. Pulmonary hypertension was associated with increased Rho-kinase activity in the third postnatal week, and Rho-kinase inhibitor treatment for 1 week tended to attenuate RVH. These results demonstrate that Rho-kinase plays an important role in the pathogenesis of hyperoxia-induced pulmonary hypertension and that Rho-kinase might be a potential therapeutic target in attenuating hyperoxia induced structural changes in pulmonary hypertension.

Author contribution

Hsiu-chu CHOU performed histological experiments, analyzed the data, and was responsible for image analysis; Liang-ti HUANG performed animal experiments; Tsu-fu YEH performed part of the research and analyzed the data; and Chung-ming CHEN performed research, coordinated experiments, analyzed the data, wrote the paper, and drafted the manuscript.

Abbreviations

ERM, ezrin, radixin, and moesin; MWT, medial wall thickness; p-MYPT1, phospho-myosin phosphatase target subunit 1; RVH, right ventricular hypertrophy.

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