Neonatal Hyperoxia Induces Pulmonary Hypertension and Rho-kinase Expression in Rats
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Abstract

Background: Chronic oxygen exposure induces pulmonary hypertension in newborn rats. Rho-kinase is upregulated in animal models of hypoxia-induced pulmonary hypertension and Rho-kinase inhibitors decrease the pulmonary arterial pressure. Less is known about the response of Rho-kinase in hyperoxia-induced lung injury in neonates. A rat model of hyperoxia-induced pulmonary injury was established and the expression of Rho-kinase was also assessed in the lungs of newborn rats exposed to prolonged hyperoxia.

Methods: Experimental rat pups were exposed to 1 wk of > 95% O₂ and a further 2 wk of 60% O₂. Control pups were exposed to room air over the same periods. Lung tissues were obtained for biochemical and histochemical assays on postnatal Day 21.

Results: Hyperoxia significantly increased type I collagen mRNA expression and total collagen content on postnatal Day 21. Rho-kinase activity was measured as the ratio of phosphorylated ERM to ERM (ezrin, radixin, and moesin) and the ratio was significantly increased on postnatal Day 21 following hyperoxic exposure. Hyperoxic exposure for 3 wk also induced structural features of pulmonary hypertension, as indicated by increased right ventricular hypertrophy and arterial medial wall thickening.

Conclusions: The results suggested that Rho-kinase might be involved in the pathogenesis of hyperoxia-induced pulmonary hypertension. (J Pediatr Resp Dis 2013;9:59-64)

Key words: Collagen, hyperoxia, pulmonary hypertension, Rho-kinase.

INTRODUCTION

Prolonged oxygen therapy exposure can cause hyperoxic lung injury in neonates. 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 (BPD).¹,² In humans, BPD remains a major cause of morbidity and mortality during the first year of life; it is characterized by arrested alveolar and vascular development, and in the advanced stage, pulmonary hypertension and right ventricular hypertrophy.³ 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.⁴,⁵ Chronic oxygen exposure induces pulmonary vascular remodeling and pulmonary hypertension in newborn rats.⁶-⁸

The small GTP-binding protein Rho and its downstream effector Rho-kinase play an important role in regulating vascular smooth muscle tone.⁹ The Rho-kinase system is constitutively active in regulating vasoconstrictor tone, and upregulation of this pathway
occurs in various cardiovascular diseases.\textsuperscript{10,11} 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.\textsuperscript{12,13} Rho-kinase has been identified as a potential therapeutic target in pulmonary hypertension.\textsuperscript{14} However, less is known about the response of Rho-kinase in hyperoxia-induced lung injury. This study tested the hypothesis that neonatal hyperoxia induces pulmonary hypertension and is accompanied by an increase in Rho-kinase expression in rat lungs.

\textbf{METHODS}

\textbf{Animals and Exposure to Hyperoxia}

The study was performed in accordance with guidelines provided and approved by the Animal Care Use Committee of Taipei Medical University. 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, n=9) or room air (control group, n=12). 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 3 wk. The hyperoxia groups were exposed to > 95\% O\textsubscript{2} for 1 wk and were then placed in an environment with 60\% O\textsubscript{2} for a further 2 wk. Oxygen exposure was carried out in a modified controller for the basic model (NexBiOxy, Hsinchu, Taiwan). Lung and heart from rats exposed to room air and hyperoxia were harvested on postnatal Day 21.

\textbf{Total Collagen Content}

Lung collagen was determined by assaying the total soluble collagen using the Sircol collagen assay kit according to the manufacturer’s instructions (Biocolor Ltd., Newton Abbey, UK).

\textbf{Reverse-Transcription (RT) and Real-Time Polymerase Chain Reaction (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). A real-time quantitative PCR was performed using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primer sequences were as follows: type I collagen sense 5’-CAACCTCAAGAAGTCTGC-3’, antisense 5’-AGGTGAATCGACTTGGCCT-3’; GAPDH sense 5’-CTCCCTCAAGATTGTCAGCAA-3’, antisense 5’-GTCAGATCCAAACGGGATCATTT-3’. The messenger (m) RNA levels were normalized to GAPDH as an internal control.

\textbf{Lung Rho-kinase Activity}

Lung protein expressions for total and phosphorylated ERM protein (a substrate for Rho-kinase) were analyzed by Western blotting. 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.\textsuperscript{12} The primary antibodies used in this study were goat anti-p-ERM (Santa Cruz, 1:200), 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 blot bands using AIDA software (Advanced Image Data Analyzer; Raytest Izotopenmessgeraete, Straubenhardt, Germany).

\textbf{Histological Examination}

The rat lung tissues were dehydrated in alcohol, cleared in xylene, and embedded in paraffin. Seven \(\mu\)m thick sections were stained with Masson trichrome (Sigma-Aldrich).

\textbf{Immunohistochemistry for p-MYPT1}

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)
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Antibodies (1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as the primary antibody. The sections were then treated with biotinylated rabbit anti-goat IgG (1:200, Jackson ImmunoResearch Laboratories 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.

**Figure 1.** (A) Type I collagen mRNA expression and (B) total collagen, and (C) Masson’s trichrome stain (×400) were determined in 21-day-old rats. Rats were exposed either to room air or hyperoxia from birth (Data are mean ± SD, *p < 0.05, **p < 0.01 compared with room air group). Hyperoxic exposure for 21 d significantly increased type I collagen mRNA expression and total collagen compared with control rats. Pulmonary fibrosis was further verified by Masson trichrome staining.

**Figure 2.** Rho-kinase activity in lung tissue in 21-day-old rats exposed to room air or hyperoxia from birth (Data are mean ± SD, **p < 0.01, compared with control group). (A) Representative Western blots for phosphorylated (p) ERM proteins and total ERM, using β-actin as a protein loading control. (B) Scanning densitometry results for quantification of the protein, with correction for β-actin levels (Data are mean ± SD, **p < 0.01 compared with room air group). Rho-kinase activity was significantly increased in hyperoxia group compared with normoxic group on postnatal Day 21.

**Figure 3.** Representative photomicrographs of p-MYPT1 (x 200). 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 endothelial cells of the vessels (v; arrow) and was enhanced in the lung tissues of hyperoxia-exposed rats on postnatal Day 21. Decreased p-MYPT1 immunoreactivity was observed in the control rats.
RESULTS

Body Weight, Heart Weight, and Heart/Body Weight Ratio (%) in Rats

The effects of hyperoxia on the rats’ body weight, heart weight, and heart/body weight ratio (%) are shown in table 1. Rats exposed to hyperoxia exhibited significantly lower body weights compared with room-air controls on postnatal Day 21. Heart weight and heart/body weight ratios were comparable between the experimental and control groups on postnatal Day 21.

Type I Collagen mRNA Expression and Total Collagen

Hyperoxic exposure for 21 d significantly increased type I collagen mRNA expression (Figure 1a). Hyperoxia-exposed rat lungs displayed a significant increase in total collagen compared with control rats on Day 21 (Figure 1b). Furthermore, pulmonary fibrosis was verified using Masson trichrome staining (Figure 1c).

DISCUSSION

Our in vivo model showed that exposure of neonatal rats to hyperoxia for 3 wk caused lung fibrosis and induced pulmonary hypertension, as shown by increased lung collagen levels and increased RVH and arterial MWT. These phenomena were associated with increased Rho-kinase expression. These results suggested that Rho-kinase might be involved in the pathogenesis of hyperoxia-induced pulmonary hypertension, and that Rho-kinase inhibitor might be a potential therapeutic target for treating hyperoxia-
induced pulmonary hypertension.

Murine alveolar development begins on postnatal Day 4 and sacculary division is completed by the fourteenth day. 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 wk of gestation. During the first 3 wk of this study, the body weight of experimental rats was approximately 80% that of the controls; heart weight was less severely affected than body weight.

In humans, newborns with respiratory disorders often receive therapy with hyperoxia. However, supplemental oxygen administered to these infants can increase their oxidant stress and lead to death of the lung epithelial cells. Failure to modify these processes may lead to excessive cell growth and proliferation, collagen overproduction, and pulmonary fibrosis. Masson’s trichrome differentiates collagen from smooth muscle and elastin, and allows the visualization and quantification of the extent of airway fibrosis. In this study, Masson’s trichrome staining resulted in collagen presenting as a dense blue-tinged material, shown surrounding the small and large airways and perivascular interstitium (Figure 1c).

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. Rho/Rho kinase-mediated Ca2+ sensitization is a key component in the sustained vasoconstriction induced by G protein-coupled receptor agonists. Kondrikov et al. reported that RhoA activation is responsible for the increase in collagen-I synthesis in hyperoxic lung fibroblasts and mouse lungs. However, it is not yet known whether RhoA is activated in pulmonary hypertension induced by oxygen toxicity. In summary, we found that exposing neonatal rats to hyperoxia caused lung fibrosis and induced pulmonary hypertension. Pulmonary hypertension was associated with increased Rho-kinase expression in the third postnatal week. These results suggest that Rho-kinase might be involved in the pathogenesis of hyperoxia-induced pulmonary hypertension, and that Rho-kinase inhibitor might be a potential therapeutic target in treating hyperoxia-induced pulmonary hypertension.

**REFERENCES**

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