Muscularization of pulmonary artery and RhoA/ROCK levels in rats exposed to intermittent hypoxia

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Abstract: Objective: Intermittent hypoxia (IH), as the most important pathophysiologic characteristic of obstructive sleep apnea (OSA) and a recognized risk factor for cardiovascular disorders, may lead to proliferation of smooth muscle cells (SMCs) and then pulmonary arterial remodeling through the RhoA/ROCK signaling pathway. In this preliminary study, an IH animal model was developed, and the muscularization of small pulmonary arteries and RhoA/ROCK levels in these rats exposed to IH were studied. Methods: Model rats were exposed to IH [cycles of hypoxia (30 s of 5% O2) and normoxia (90 s of 21% O2)] or intermittent normoxia [cycles of normoxia (30 s of 21% O2) and normoxia (90 s of 21% O2)] for 4 weeks, 9 AM to 5 PM in every day. Immunohistochemistry of α-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) were used to measure muscularization and status of cell proliferation in small pulmonary arteries. Real-time polymerase chain reaction (RT-PCR) and Western blot analyses were used to assess levels of RhoA and ROCK expression. Results: A significantly increased ratio of α-SMA positive muscularized vessels and higher proliferative rate were observed in IH group compared with Control group (P < 0.05). Levels of RhoA/ROCK mRNA and protein in IH group were significantly higher than those in Control group (P < 0.05). Conclusion: Our preliminary data implicated that IH may induce proliferation of SMCs and then pulmonary arterial remodeling through the RhoA/ROCK signaling pathway, which may involve the development of PH.

Keywords: Intermittent hypoxemia, pulmonary hypertension, muscularization, RhoA/ROCK

Introduction

Obstructive sleep apnea (OSA) is a kind of sleep-disordered breathing, characterized by complete pharyngeal closure (apnea) or partial closure (hypopnea) during sleep due to loss of tone of the upper airway muscles. Repetitive episodes of apnea or hypopnea result in arterial hypoxia, hypercapnia, surge in pulmonary blood pressure due to pulmonary vasoconstriction and pulmonary arterial remodeling [1]. Pulmonary hypertension (PH), characterized by an elevated, sustained increase in pulmonary artery pressure greater than 25 mmHg at rest or 30 mmHg upon exertion, is a progressive disease with poor prognosis and death usually occurs within 5 years if left untreated [2]. When exposed to intermittent hypoxia (IH) for several hours per day to mimic OSA, animals have shown to develop sustained PH and pulmonary vascular remodeling within a few weeks [3, 4]. The typical pathological changes of PH include pulmonary vascular constriction, pulmonary vascular remodeling (muscularization and thickening of precapillary pulmonary arteries, intimal proliferation, obliterative lesions), and thrombosis in situ [5]. Pulmonary arterial muscularization, the key component of pulmonary vascular remodeling, is characterized by uncontrolled and inappropriate proliferation of pulmonary arterial smooth muscle cells (PASMCs) [6].

The activation of small GTPase, small G-protein RhoA, and its effector protein ROCK is strongly implicated as a key pathway involved in the sustained pulmonary vasoconstriction and vascular remodeling, which contributes to PH consequently [7]. Recent studies have revealed that
RhoA/ROCK signaling pathway is associated with increased pulmonary artery expression of growth factors and markers of cell proliferation, matrix protein production, and inflammatory cell infiltration [8, 9]. In addition, it is reported that this pathway regulates reconstruction of microfilaments of PASMCs by mediating myosin light chain (MLC) phosphatase and activating of downstream mediators [10]. Furthermore, in vitro studies suggest that ROCK inhibitors are effective treatments for severe PH with minimal risk, supporting the premise that ROCKs are important therapeutic targets for PH [11, 12].

We hypothesize that IH, as the most important pathophysiologic characteristic of OSA and a recognized risk factor for cardiovascular disorders, may lead to proliferation of PASMCs and then pulmonary arterial remodeling through the RhoA/ROCK signaling pathway. In this preliminary study, an IH animal model was developed, and the muscularization of small pulmonary arteries and RhoA/ROCK levels in these rats exposed to IH were studied.

Materials and methods

Animals

Institutional Review Board of Tianjin Medical University General Hospital approved the ethical and methodological aspects of the investigation (TMU IRB Approving Number: EA-20-120002). Thirty male Wistar rats weighing 120~150 g at age of 4 weeks (provided by Model Animal Center of Radiological Medicine Research Institute, Chinese Academy of Medical Science, license No.: SCXK Tianjin 2006-0009) were divided into Control group (sham IH exposure, n = 15) and IH group (true IH exposure, n = 15) according to exposure conditions. Animals were housed in standard laboratory cages (5 per cage) and allowed food and water ad libitum.

Animal IH exposure

Model rats were exposed to IH for 4 weeks, 9 AM to 5 PM in every day. We used an IH exposure device the same with that seen in one of our previous studies [13]. Briefly, a gas control delivery system was designed to regulate the flow (5 L/min) of nitrogen (N₂, IH phase) or clean air (air, reoxygenation phase, ROX phase) alternatively into a customized IH housing chamber to maintain a designated IH (30 s) or normoxia (90 s) environment, producing 30 cycles of alternations per hour.

Preliminary experiment to get arterial blood gas (ABG) values

Two days before the end of exposure period, 5 rats were selected randomly from each group for obtaining ABG data. After anesthetization, the right femoral artery of selected rats was cannulated to obtain blood samples for monitoring ABG at any time as necessary. At the end of treatment, arterial blood samples were drawn and ABG values were measured at the following time points: Point 1, during exposure time (9 AM to 5 PM) with clean air exposure in Control group; Point 2, nadir partial oxygen pressure (PaO₂) point during hypoxia phase in IH group with IH exposure; Point 3, zenith PaO₂ point during ROX phase in IH group with IH exposure.

Preparation of lung samples

The animals were anesthetized with isoflurane and thoracotomy was operated to expose the lung. The right lung was removed, fleshly frozen in liquid nitrogen and stored at -80°C for later use. The left lung was then removed en bloc from the body following inflation fixation of the lung with formalin under 30 cm H₂O pressure and stored in paraformaldehyde.

α-smooth muscle actin (α-SMA) staining of lung sections for analyses of muscularization

Left lungs were transversely cut into 4 μm-thick tissue slices. Paraffin-embedded lung sections were baked 60 min at 55°C, deparaffinized in xylene and rehydrated through decreasing alcohol concentrations gradually. Sections were boiled in antigen unmasking solution (Vector laboratories, H-3300, Burlingame, CA) for 10 minutes and blocked using 2.5% horse serum (Vector Laboratories, Burlingame, CA) followed by incubated overnight at 4°C with mouse monoclonal α-SMA antibody (1:100, Invitrogen, Carlsbad, CA). The following day, sections were incubated with anti-mouse secondary antibody (1:200, Invitrogen, Carlsbad, CA). Nuclei were counterstained with hematoxylin and slides were analyzed at 400 x using Olympus BX 40 microscope (Olympus optical co. Ltd., Tokyo, Japan).
Table 1. Descriptive ABG results from preliminary tests

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IH-H</th>
<th>IH-R</th>
</tr>
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<tbody>
<tr>
<td>$\text{PaO}_2$ (mmHg)</td>
<td>94.300±2.6106</td>
<td>51.520±1.1798</td>
<td>90.220±4.0301</td>
</tr>
<tr>
<td>$\text{PaCO}_2$ (mmHg)</td>
<td>41.860±2.3373</td>
<td>37.020±0.9149</td>
<td>40.740±3.2098</td>
</tr>
<tr>
<td>$O_2\text{Sat}$ (%)</td>
<td>97.100±0.2915</td>
<td>86.86±1.8338</td>
<td>95.300±1.6294</td>
</tr>
<tr>
<td>pH</td>
<td>7.41100±0.009434</td>
<td>7.41980±0.009731</td>
<td>7.41640±0.011216</td>
</tr>
</tbody>
</table>

PaO$_2$: arterial oxygen partial pressure; PaCO$_2$: arterial carbon dioxide partial pressure; O$_2$Sat: arterial oxyhemoglobin saturation; IH-H: hypoxia phase in IH group; IH-R: ROX phase in IH group.

To determine the degree of muscularization, vessels < 100 μm with positive α-SMA cell staining surrounding endothelial cells were identified and classified as fully muscularized (100~75%) and partially muscularized (< 75%). Vessels with no significantly positive α-SMA staining were classified as “non-muscularized”. When we chose the examined microscopic fields for vessels < 100 μm, the including criteria was about 90 μm for outer diameters of the vessels. The degree of muscularization was expressed as % ratio of the number of muscularized to the number of total observed cells.

**Proliferating cell nuclear antigen (PCNA) staining of lung sections**

PASMC and endothelial cell proliferation were evaluated with immunochemical staining of PCNA. Four-μm sections from paraffin embedded blocks were treated with three changes of xylene to remove paraffin wax, followed by descending grades of alcohol and rehydration with water. After transferred to citrate buffer and autoclaved for antigen retrieval, the slides were washed in PBS and blocked with 3% hydrogen peroxide and protein blocking reagent respectively. The slides were incubated with primary antibody against PCNA (1:100, Dako, Carpinteria, CA, USA) for 45 min; subsequently, the sections were incubated with a biotinylated anti-mouse antibody for 30 min. Nuclei were counterstained with hematoxylin and slides were analyzed at 1000 X using Olympus BX 40 microscope (Olympus optical co. Ltd., Tokyo, Japan). The brown stained nuclei of smooth muscle or endothelial cells were considered as positive. Thus the percentage of proliferating smooth muscle or endothelial cells in PCNA immunostained slides were evaluated and expressed as % ratio of the positive smooth muscle or endothelial cell number to the number of total observed cells.

**RhoA and ROCK mRNA expression measurement**

The right lung, frozen in liquid nitrogen and stored at -80°C, was used for mRNA expression measurement. Lung tissue blocks were lysed in Trizol (Invitrogen, Carlsbad, CA) and total RNA was extracted. cDNA was synthesized from 2 μg of total RNA by incubation at 42°C 50 min, 95°C 5 min with avian myeloblastosis virus reverse transcriptase (GE Healthcare, Little Chalfont, UK) and random hexanucleotides according to the manufacturer’s instruction. Sequences of primers used to specifically amplify the genes were as follows: RhoA, 5’-AGGACCAGTTCCAGAGGT-3’ (forward) and 5’-TAGGAGAGGCCCTCAGCG-3’ (reverse); ROCK, 5’-GAGATCAGTGCAGCGGCTAT-3’ (forward) and 5’-CACTACCAGCTTTGAGGT-3’ (reverse); GAPDH, 5’-TGGAGTCTACTGGCGTCTTC-3’ (forward) and 5’-TTCACACCCATCACAAACATG-3’ (reverse). The Real-time polymerase chain reaction (RT-PCR) assay was performed using SYBR Green One-Step RT-PCR Kit (Invitrogen-Life Technologies, Carlsbad, CA), according to manufacturer’s recommendation. The reaction was prepared in a 96-well plate format, using 1 μl of cDNA template and 0.5 μl of each primer in a final volume of 10 μl. Thermal cycle settings consist of 5 minutes of Taq polymerase activation at 95°C, 10 seconds of denaturation step at 95°C and 30 seconds of annealing and extension steps at 72°C. The results were standardized with internal reference, GAPDH mRNA, and expressed as a percentage.

**Western blot studies**

Frozen rat lung tissue was pulverized with a mortar and pestle, resuspended and homogenized. The tissue homogenate was centrifuged at 3000 rpm for 15 min. Protein concentration was determined by Bradford assay (Bradford reagent, Sigma, St. Louis, MO). For Western blot analysis, the cell lysates were separated by 12% SDS-PAGE, transferred into a polyvinylidene fluoride membrane (GE Healthcare, Little Chalfont, UK), blocked with 5% skim milk, and
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incubated with the primary antibodies (1:1000, RhoA and ROCK, Cell Signaling, Danvers, MA; GAPDH, EarthOx Company, San Francisco, USA) overnight. Afterwards, membranes were incubated with the secondary antibodies (1:5000) at room temperature for 1 hour and the protein bands of interest were identified using enhanced chemiluminescence reagent. The results were standardized with internal reference, GAPDH protein, and expressed as a percentage.

Statistical analysis

Data were reported as mean ± standard deviation (SD). Independent-sample t-test was used to evaluate differences between IH and Control groups. P < 0.05 was considered statistically significant.

Results

Table 1 summarized ABG results obtained from preliminary experiments two days before the end of exposure period. The values in this table are descriptive, not for comparing between groups. PaO₂ value in Control group is 94.300±2.6106 mmHg. PaO₂ value of IH group in hypoxia phase and ROX phase of slow wave sleep period is 51.520±1.1798 and 90.220±4.0301 mmHg, respectively.

Evaluation of muscularization

Compared with Control group, IH exposure increased the number of α-SMA positive profiles observed per microscopic field (Figure 1A). Of total observed blood vessels, the ratio of muscularized vessels was 35.44±1.41% in Control group, when this ratio was increased to 42.66±1.63% in IH group (P < 0.05) (Figure 1B).

PASMC and endothelial cell (EC) proliferation

PASMC and EC proliferation was evaluated by PCNA immunohistochemistry. A significantly higher proliferative rate was observed in IH group compared with Control group, where we occasionally found proliferating cells (Figure 2A). Quantitative analysis performed on targeted small pulmonary arteries (5 slides/rat, 15 rats/group) showed that there were 54.67±1.80% PCNA positive cells in IH group when only 9.14±0.91% PCNA positive cells were observed in Control group (P < 0.05) (Figure 2B).

Pulmonary RhoA mRNA and ROCK mRNA expression

As shown in Figure 3, IH exposure resulted in significantly higher RhoA mRNA and ROCK mRNA expression levels than those in Control group (0.463±0.067 vs 0.182±0.040 for RhoA and 0.384±0.062 for ROCK, P < 0.05).
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Pulmonary RhoA and ROCK protein expression

IH exposure increased RhoA protein expression levels to 0.827±0.065 from 0.424±0.075 in Control group (P < 0.05) and increased ROCK protein expression levels to 0.488±0.088 from 0.336±0.102 in Control group (P < 0.05), as shown in Figure 4 of Western blot results.

Discussion

In an attempt to better understand mechanisms of vascular dysfunction related to OSA-associated derangements, our study employed a rat model of IH, which is the most important pathophysiologic characteristic of OSA. Consistent with previous reports that IH in the rat leads to PH [14, 15], our results revealed that IH-induced PH was associated with increased muscularization and PASMC/EC proliferation, resulting in remodeling of pulmonary vasculature. RhoA/ROCK is an appealing target for the development of PH as many studies have shown that RhoA/ROCK signaling pathway may be involved in the sustained vasoconstriction and vascular remodeling [16, 17].

In previous studies, when exposed to IH for several hours per day to mimic OSA, animals have been shown to develop sustained PH and pulmonary vascular remodeling within a few weeks [3, 4]. The typical pathological changes of PH include pulmonary vascular constriction, pulmonary vascular remodeling (muscularization and thickening of otherwise non-muscularized distal pulmonary arteries, progressive intimal proliferation, obliterative lesions), and thrombosis in situ [5]. Pulmonary vascular remodeling which leads to increased pulmonary vascular resistance and reduced compliance, is characterized by pulmonary arterial medial hypertrophy and muscularization due to uncontrolled and inappropriate proliferation of PASMCs [6], though the mechanisms left unclear. Hyperplasia of PASMCs may partly be resulted from PASMCs’ phenotype alteration into a contractile and “differentiated” phenotype by expressing a number of smooth muscle-specific genes and proteins, including SMA, which contributes to the contractile function of PASMCs [18, 19]. So the increased muscularization may be visualized by α-SMA staining in lung sections. Our results showed that expression of α-SMA in pulmonary vasculature, a marker of early smooth muscle differentiation, was significantly higher in IH group than that in Control group, suggesting a potential switch of PASMCs towards a proliferative phenotype. The
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proliferation of PASMCs in PH driven by many factors including the *de novo* expression of the anti-apoptotic protein surviving [20], the increased expression/activity of the serotonin transporter [21] and the increased expression/activity of platelet derived growth factor receptor, is enhanced, whereas apoptosis is inhibited [20, 22].

PCNA levels are elevated in the S, G2, and M phases of cell mitosis and are often used as a marker for proliferation. Dedifferentiation or phenotypic modulation of SMCs has been widely depicted in various vascular wall diseases including atherosclerosis [23] and enhanced proliferation of distal precapillary PASMCs and PAECs in vitro has also been described in PH [24]. Mushaben and co-workers showed that persistent inflammation could induce pulmonary vascular remodeling and the PH might be partly due to a higher percentage of muscularized pulmonary arteries [25]. The significantly higher PCNA levels of IH group in this study confirmed a switched proliferative phenotype of PASMCs during IH exposure.

RhoA/ROCK signaling pathway is an important regulator of vascular tone along with inflammation, hyperglycemia, and oxidative stress [26]. Multiple cytokines and inflammatory mediators [27], through G-protein coupled receptors, activate guanine nucleotide exchange factors, which induce exchange of GDP for GTP binding and translocation of GTP-RhoA to the plasma membrane. RhoA combines with downstream target molecule ROCK to regulate a variety of cellular functions including motility, proliferation, apoptosis, contraction, and gene expression [28], and is believed to be the most important regulators of Ca\(^{2+}\) sensitivity in smooth muscle [29]. Phosphorylation (contraction) and dephosphorylation (relaxation) of the regulatory myosin light chain (MLC) in SMCs is catalysed by Ca\(^{2+}\)/calmodulin-dependent MLC kinase (MLCK) and Ca\(^{2+}\)-independent MLC phosphatase (MLCP) that is targeted to myosin by its regulatory myosin binding subunit. At a given level of cytosolic Ca\(^{2+}\), second messenger-mediated pathways can modulate the activity of both enzymes to modify MLC phosphorylation and the Ca\(^{2+}\) sensitivity of contraction. And two major pathways of Ca\(^{2+}\) sensitization in SMCs are inhibition of MLCP by ROCK-mediated phosphorylation of regulatory myosin-binding subunit, and protein kinase C (PKC)-mediated phosphorylation and activation of the MLCP-inhibitor protein PKC-potentiated inhibitor protein of 17 kDa (CPI-17) [30]. In addition, ROCK can also phosphatase myosin-binding subunit myosin light chain phosphatase (MYPT-1) and/or the MLCP inhibitor protein CPI-17. Evidence from in vivo studies demonstrates that the effect of ROCK inhibitors is associated with decreased pulmonary artery expression of growth factors and markers of cell proliferation, matrix protein production, and inflammatory cell infiltration as well as an increase in signals for apoptosis, suggesting the opposite effects on ROCK of vasoconstriction and vascular remodeling [31]. Liu and his colleagues reported that activation of RhoA and ROCK, and ROCK-mediated translocation of phosphorylated extracellular signal-regulated kinase to the nucleus are involved in serotonin induced proliferation of bovine PASMCs, depending on multiple serotonin receptors and transporters [32].

In previous studies, administration of vasodilators only partially reversed the increase in pulmonary arterial pressure secondary to chronic

Figure 3. A. RhoA mRNA expression was significantly higher in IH lung samples than Control. B. ROCK mRNA expression was significantly higher in IH lung samples than Control. *P < 0.05 when compared between IH and Control groups.
hypoxia (CH) [33, 34], suggesting a large fixed component of hypoxic PH also partly due to proliferation of SMC. A study demonstrated that there was an enhanced cell proliferation in human PASMCs stimulated with PDGF, founding that the percentage of cells in S phase increase from 15.6 to 24.3% and significantly upregulated ROCK activity [35]. Prolonged therapy with fasudil, a Rho kinase inhibitor, in rats with PH led to significantly reduced ROCK activity over 8 weeks, improved pulmonary vascular hemodynamics and suppressed the proliferation of the PASMCs and vascular remodeling [36].

Exposure to CH has been shown to increase pulmonary arterial depolarization-induced superoxide (O$_2^-$) generation and O$_2^-$ stimulates RhoA/ROCK signaling and mediates enhanced RhoA-dependent Ca$^{2+}$ sensitization in vascular SMC [37]. Oxidative stress, occurring via activated inflammatory responses induced by hypoxia and by an increased sympathetic activity, is a known feature of OSA [38] and is thought to be mainly caused by cyclical hypoxia/reoxygenation. So we speculate that similar with CH, IH may also increase O$_2^-$ generation and further mediate enhanced RhoA-dependent Ca$^{2+}$ sensitization in SMCs.

In the present study, mRNA expression of RhoA and ROCK was significantly upregulated in IH group compared with Control group, so was RhoA and ROCK protein expression. Pulmonary vasoconstriction and vascular remodeling is considered to be the leading cause of PH. A study showed that patients without any other lung or heart disease and characterized by older age, greater obesity and lower daytime oxygenation developed mild PH and had significantly decreased oxygen partial pressure compared to patients without PH [39]. We speculate that IH alone constitutes a risk factor for the development of PH. Evidence strongly suggests that RhoA/ROCK is an attractive target for the development of therapeutics to treat PH as studies have shown that RhoA/ROCK signaling pathway is involved in the sustained vasoconstriction, vascular remodeling, and PH finally.

Several limitations of the present study are acknowledged. First, a study shows that decreasing the alveolar oxygen pressure to < 70 mmHg elicits a strong pulmonary vasoconstrictor response; however, the hypoxic-induced effect varies among animal species [40], and hypoxic pulmonary vasoconstriction is milder in humans than in rats [41]. Thus, caution must be raised when extrapolating animal models of chronic hypoxic-induced PH to the human setting. Second, as IH constitutes a risk factor for the development of PH, these should undoubtedly be clarified by additional measurements of pulmonary vascular hemodynamics. Hemodynamics was not considered in this study because we thought that muscularization is a leading unlied source of PH and it may not be needed in a mechanism study. Third, pulmonary arterial thickening and muscularization are two prominent pathological features for PH.
42. Medial thickening is characterized by increased smooth muscle mass, which results from alterations in SMC phenotype and function, and contributes significantly to increased PA resistance [42]. The appearance of new smooth muscle in previously non-muscular or partially muscular vessels is termed as muscularization. Mature vascular SMCs express a number of smooth muscle-specific genes and proteins characteristic of their contractile and “differentiated” phenotype, mainly including SMA, which contribute to the contractile function of SMC [43]. Although myogenic tone can be increased by vessel wall hypertrophy through the “structural amplifier” mechanism [44], in this article, functional rather than structural mechanisms account for PH was more concerned. That’s the reason why the degree of muscularization, such as medial wall thickness, was not measured. Fourth, RhoA/ROCK signalling has not only been implicated in mediating sustained, abnormal vascular SMC contraction, but also in several other processes [45, 46], and PCNA levels, which elevated in the S, G2, and M phases of cell mitosis, are also expressed in other cell types. We used the whole lung samples in our study because we couldn’t definitely separate these individual cell types, and this design flaw may be figured out in future cellular level studies.

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Disclosure of conflict of interest

None.
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