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Full Paper

Time-Dependent Phenotypic and Contractile Changes of Pulmonary Artery in Chronic Hypoxia–Induced Pulmonary Hypertension

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Abstract. Phenotypic and contractile changes in pulmonary arterial smooth muscle cells (PASMCs) were examined in rats with pulmonary hypertension induced by hypoxia. Exposure to hypoxia induced pulmonary hypertension within 1 – 4 weeks. Staining with BrdU revealed that proliferative activities of PASMCs peaked at 1 week of hypoxic exposure, and then moderate proliferative activity was maintained for the next 2 – 4 weeks. The β-actin/α-actin ratio also increased at 1 – 2 weeks of exposure to hypoxia. Absolute contractility of the pulmonary arterial ring continuously decreased during hypoxia, whereas the basal active tonus of the pulmonary artery increased at 1 – 3 weeks. Nicardipine, the ET_A-receptor antagonis, CI-1034 and the rho-kinase inhibitor Y27632 partially inhibited the elevated active tonus. Endothelin-1 content in the pulmonary hypertensive lung was continuously increased during exposure to hypoxia. In conclusion, the hypoxia-induced proliferative activity of PASMCs comprised a transient phase followed by a sustained phase. The change in PASMCs from a contractile to a synthetic phenotype also correlated with proliferative activity, which subsequently decreased PASMC contractility. The continuous production of endothelin-1 upon hypoxic exposure might contribute to the increased basal tonus of the pulmonary arterial wall, which might subsequently increase pulmonic arterial pressure, resulting in accelerated pulmonary hypertension.

Keywords: pulmonary artery, hypoxia, contraction, endothelin-1, proliferation

Introduction

Pulmonary hypertension (PH) is characterized by elevated pulmonary blood pressure without a change in the systemic circulation and it is categorized as primary or secondary types. The pathogenesis of PH comprises sustained vasoconstriction (1), structural remodeling of the pulmonary artery, and thrombosis (2). Chronic hypoxia is considered a critical factor that causes sustained PH and aggravates pathophysiological conditions. Furthermore, continuous alveolar hypoxia causes PH that could lead to right heart failure and death. However, the mechanism governing the development of this syndrome remains unclear. Consequently, current options for effective prevention and therapy are limited.

Vascular smooth muscle cells (VSMC) comprise mainly "contractile" and "synthetic" types (3). Contractile-type VSMCs have spindle-shape nuclei and are arranged in laminae. These cells are rich in α-actin, myosin heavy chain (MHC) type SM-1 and SM-2, SM22α, vinculin, and meta-vinculin, resulting in high contractile ability (4). However, the synthetic-type can be found among undifferentiated VSMCs such as those of newborns (fetus) and the vascular system during angiogenesis. Synthetic VSMCs are characterized by low contractility and high proliferation activities, minimal amounts of myofibrillar protein, enriched protein-synthetic subcellular organelles (5, 6), and the presence of β-actin and non-muscular myosin heavy chain (NMHC) types A and B. Synthetic VSMCs are thought to play an important role in the development of several vascular diseases of humans such as atherosclerosis, restenosis, and hypertension (7). However, the role of phenotypic changes in pulmonary arterial smooth muscle cells (PASMCs) in the development of hypoxia-induced PH remains vague.

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Changes in pulmonary vasoconstriction are important in the development of PH and vascular remodeling (8). Many factors like vasoconstrictors, such as endothelin-1, angiotensin II, and serotonin, and vasodilators, such as nitric oxide and prostacyclin, can regulate pulmonary vasoconstriction during chronic hypoxia. Endothelin-1 contributes to the pathogenesis of hypoxic PH (9 – 12). Chronic hypoxia increases plasma endothelin-1 levels (13, 14) and selective endothlin-1–receptor antagonists prevent the progression of PH (14, 15). Conversely, we reported that the loss of nitric oxide production in endothelial cells during chronic hypoxia is critical in the induction of PH (16, 17). In addition, chronic hypoxia directly affects the contractile ability of pulmonary smooth muscle cells (18, 19). However, the phenotype of contractility is erratic in the pulmonary artery of PH (20 – 22). This might be due to different pathogenetic stages, choice of animal model, and branch of the pulmonary artery. In the present study, we investigated time-dependent changes in the phenotype of PASMCs and contracture of main pulmonary artery in rats exposed to chronic hypoxia.

Materials and Methods

Experimental groups and chronic hypoxia

Chronic PH was induced by hypoxia in rats as described by Murata et al. (16, 23). Male Sprague-Dawley rats (Charles River Breeding Laboratories, Yokohama) at 7 weeks of age were randomly separated into one group that was maintained in room air (20% O_2: normoxia) and another that was exposed to normobaric hypoxia (10% O_2) in transparent plastic chambers for 1 – 4 weeks. Relative humidity and minimal NH_3 levels within the chambers were maintained using anhydrous $CaSO_4$ and boric acid, respectively. One chamber was flushed with nitrogen to establish a hypoxic environment that was monitored using an oxygen analyzer (MB1000; Chino Corporation, Tendo). The chamber was maintained at 22°C – 24°C, and it was opened every day for 10 min to clean the cage and replenish the standard rat chow and water that were provided ad libitum. Normoxic rats (210 – 240 g) were housed for 1 – 4 weeks under identical conditions including a 12 h : 12 h light–dark cycle, except for breathing room air. Animal experiments and care were approved by local ethical review (approval code: 1515T0007) and were performed in strict compliance with the guidelines outlined within the *Guide to Animal Use and Care from the University of Tokyo* and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Tissue preparation

After exposure to hypoxia or normoxia for 1 – 4 weeks, the rats were weighed, sacrificed by administering an excess of diethylether anesthesia, and then the heart and lungs were removed *en bloc* via a thoracotomy. The right and left main outer pulmonary arteries (<2.0-mm lumen diameter) were dissected and cut into 1-mm rings. The right ventricle (RV) and left ventricle plus septum (LV+S) were then separated and weighed to investigate right ventricular hypertrophy (24).

Morphological investigation

The lungs were cut into 1-mm slices at the same point (12 mm from the top of the upper lobe of the left lung and 5 mm from the top of the lower lobe of the right lung) and placed in neutral buffer (pH 7.4) containing 10% formalin (Wako, Osaka). Paraffin-embedded lungs and pulmonary arteries were sliced into 4-μm-thick sections and stained with hematoxylin and eosin (HE). Photo images of each lung section were captured and arterial diameter was measured using an image-processing program (NIH Image 1.55; National Institutes of Health, Springfield, VA, USA) as described (16, 17). The area enclosed by the internal elastic lamina was subtracted from that bordered by the external elastic lamina to obtain the area of the medial layer, and wall thickness was calculated as the length of the medial layer.

BrdU labeling index

Immunohistochemical staining with BrdU proceeded as described by Sakamoto et al. (25). In brief, rats were injected intraperitoneally with 100 mg/kg of BrdU at 48 and 24 h before sacrifice, and 6-μm-thick sections were prepared and stained using the Vector ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). Anti-BrdU mouse monoclonal antibody (Dako, Carpinteria, CA, USA) and biotin-labeled anti-mouse IgG polyclonal antibody (KPL, Baltimore, MD, USA) served as the primary and secondary antibodies, respectively. Labeled main pulmonary arterial smooth muscle cells were identified by light microscopy (×400) and are expressed as ratios (%) of all positively stained smooth muscle cells.

Measurement of α- and β-actin ratio in PASMC

The endothelium of pulmonary arteries was stripped by gently rubbing the intimal surface with forceps and then the arteries were homogenized in protein extraction buffer: 50 mM Tris-HCl containing 50 mM NaCl, 100 mM NaF, 2 mM Na_3PO_4, 15 mM $Na_4P_2O_7$, 1% Triton-X100, 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenyl-methylsulfonyl-

fluoride (PMSF). Nuclei and non-lysed cells were removed by centrifugation at 13,500 × *g* for 10 min at 4°C. Equal amounts of protein (10 μg) from total homogenates of pulmonary arteries were separated by SDS-PAGE, and then the ratio of β-actin to α-actin in each sample was determined using anti-human smooth muscle actin clone 1A4 mouse monoclonal antibody (Dako), anti-β-actin clone AC-74 mouse monoclonal antibody (Sigma-Aldrich, Shinagawa-ku, Tokyo), and AP GxMouse IgG alkaline phosphatase (EY Laboratories Inc., San Mateo, CA, USA).

Muscle tension

The endothelium was removed from pulmonary arteries by gently rubbing the intimal surface with forceps; we confirmed that endothelium-dependent relaxation by acetylcholine disappeared. Vascular muscle tension was recorded as described (16, 17). In brief, the pulmonary arterial rings were placed in normal physiological salt solution (PSS) containing 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 5.5 mM glucose, and 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA). The PSS was saturated with a 95% O_2 – 5% CO_2 mixture at 37°C to adjust the pH to 7.4. Muscle tension was recorded isometrically with a force displacement transducer (Orientec, Tokyo) connected to a strain amplifier (Model 3134 or 3170; Yokogawa, Mitaka) and an ink-writing recorder (Model 3056 or 3711, Yokogawa) under a resting tension of 10 mN, which induced a maximum contractile force in the muscle length–tension relation curve. Griffith et al. reported that no difference was detected in the velocity of shortening or in total shortening ability in the pulmonary artery of hypoxia-induced PH (26). At the end of the experiment, papaverine (100 μM) was added to determine the basal tone. Data are shown as % relaxation of the steady-state preconstriction. The concentration of each drug (nicardipine, CI-1034, Y27632, and papaverine) was sufficient to induce the maximum pharmacological reactions.

Enzyme linked immunosorbent assay (ELISA)

The production of ET-1 was determined using the QuantiGlo ET-1 Immunoassay System (R&D Systems, Inc., Minneapolis, MN, USA) in lung tissues that had been flash frozen in liquid nitrogen and stored at −80°C. Frozen lungs were homogenized in protein extraction buffer containing 0.1 mM NaH_2PO_4, 0.1 mM Na_2HPO_4, 9% NaCl, 1% BSA, 0.1% Tween-20, 1 mM benzamidine, 1 mM PMSF, 1 mM DTT, 1 mM pepstatin A, 10 μg/ml leupeptin, and 1 μg/ml aprotinin. After centrifugation at 20,000 × *g* for 15 min, the supernatant fractions were portioned into microtiter plates coated with anti-ET-1 monoclonal antibody and then ET-1 was detected using peroxidase-labeled anti-ET-1 monoclonal antibody.

Reagents

We obtained the following reagents from the sources described in parentheses: EDTA and 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto), 4-aminopyridine (Tokyo Kasei Kogyo Co., Tokyo), DTT (Wako), papaverine hydrochloride and nicardipine hydrochloride (Sigma-Aldrich Japan), pepstatin A, leupeptin, aprotinin and PMSF (Funakoshi Co., Ltd., Tokyo) and Y27632 (Calbiochem, San Diego, CA, USA). CI-1034 was a gift from Discovery Research, Pfizer Global Research and Development, Nagoya Laboratories (Aichi).

Statistical analyses

Experimental results are shown as the means ± S.E.M. The data were statistically evaluated by the unpaired Student's *t*-test for comparisons between two groups and by the one-way analysis of variance (ANOVA) followed by Dunnett's test for comparisons among more than two groups. A value of $P<0.05$ was regarded as significant.

Results

Effect of chronic hypoxia on body weight and cardiac weight

Figure 1 shows that the rate of increase in the weight of the rats was slower from 1 week after exposure to hypoxia than in those exposed to normoxia. The loss of body weight was maintained for 4 weeks after exposure to hypoxia. Figure 2 shows changes in the ratio of the weight of the right ventricle (RV) to that of the left ventricle (LV) + interventricular septum (S). The ratio of RV / LV+S was higher in hypoxic rats, suggesting that PH induced right ventricular hypertrophy, although we did not measure pulmonary pressure directly in the present study. These results indicated that right ventricular hypertrophy is induced from 1 week after exposure to hypoxia and gradually increased until 4 weeks later.

Pulmonary arterial remodeling

Figure 2 shows the main pulmonary arterial wall stained with hematoxylin and eosin (HE) and time-dependent quantitation of the media thickness of the pulmonary arterial wall isolated from hypoxic and control (normoxic) rats. The arrangement of PASMCs in the media layer of the normoxic outer pulmonary arterial wall was orderly and the nuclei were generally

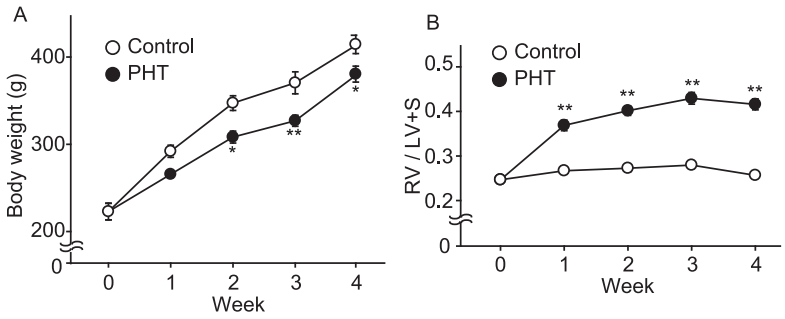

Fig. 1. Changes in body weight (A) and right ventricular hypertrophy (B) in rats with chronic hypoxia–induced PH. Right ventricle weight per left ventricle plus septum weight of the hypoxic rat is considered a marker of right ventricular hypertrophy (n = 6 – 24). All results are means ± S.E.M. **P*<0.05 and ***P*<0.01: significantly different from normoxic arteries. RV, wet weight of right ventricle; LV+S, wet weight of left ventricle plus septum. Change in body weight measured in 6 – 24 animals. Control, normal rat; PHT, pulmonary hypertensive rat.

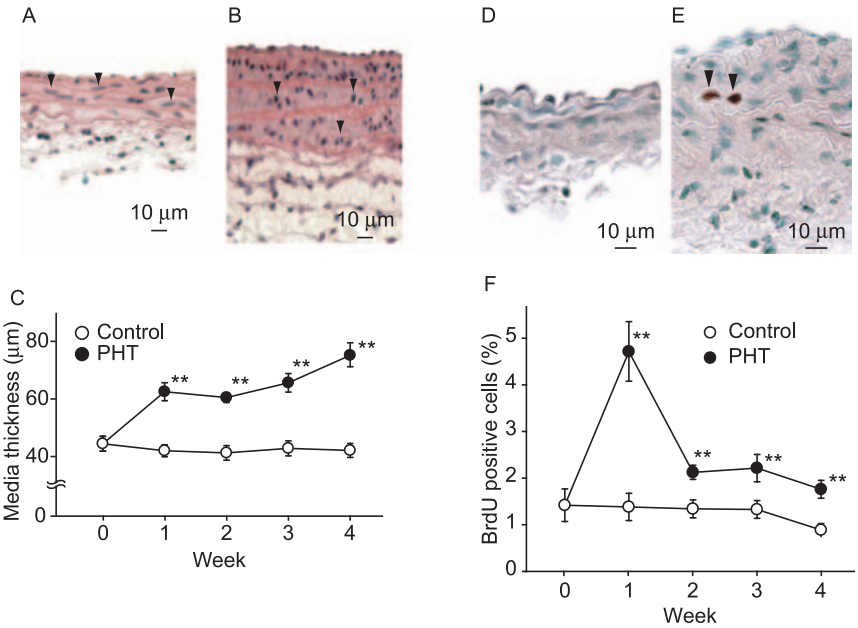

Fig. 2. Time-dependent change in media thickness of pulmonary artery (A, B, C) and proliferative activity (D, E, F) in rats with hypoxia-induced PH. Main pulmonary artery media stained with HE (n = 6 – 14) from normoxic (A) and hypoxic (B) rats at 2 weeks. Cells from normoxic rats have spindle-shaped nuclei (black arrow heads), whereas those from hypoxic rats have round nuclei (black arrow heads). C: Media thickness. All results are means ± S.E.M. ***P*<0.01: significantly different from normoxic arteries. Histological images taken at 1 week are representative of BrdU-labeled main pulmonary arteries from normoxic (D) and hypoxic (E) rats. Arrowheads (E) indicate BrdU-positive smooth muscle cells. Ratio (%) of BrdU positive cells /total cells in main pulmonary artery media (F) (n = 10 – 13). All results are means ± S.E.M. ***P*<0.01: significantly different from normoxic arteries. Control, normal rat; PHT, pulmonary hypertensive rat.

spindle-shaped. In contrast, cell orientation was lost and the nuclei were rounded in the arterial walls of hypoxic rats with PH (Fig. 2B). The thickness of the outer pulmonary arterial media wall was significantly increased within 1 week and gradually increased until 4 weeks in the hypoxic, compared with the normoxic rats (Fig. 2C). The wall thickness of the inner pulmonary arterial media plus adventitia also significantly increased from 1 to 2 weeks and continued to increase until 4 weeks (data not shown).

Figure 2, D and E, shows immunohistochemical staining of pulmonary arteries from normoxic (D) and hypoxic (E) rats. Very few nuclei were BrdU-positive in the media layer of pulmonary arteries isolated from normoxic rats. In contrast, the numbers of BrdU-positive nuclei were notably increased at 1 week after exposure to hypoxia and reduced at 2 weeks, but the level remained above that of the normoxic rats until 4 weeks later (Fig. 2F).

Changes in PASMC phenotype

We examined the expression of α-actin and β-actin proteins in the pulmonary artery by Western blotting to assess the smooth muscle phenotype. The α-actin content was enriched, whereas that of β-actin was relatively low level expression in pulmonary arteries from normoxic rats at any time between 0 and 4 weeks. The ratio of β- to α-actin remained low throughout the experimental period. In contrast the β-actin content was increased and that of α-actin was decreased in

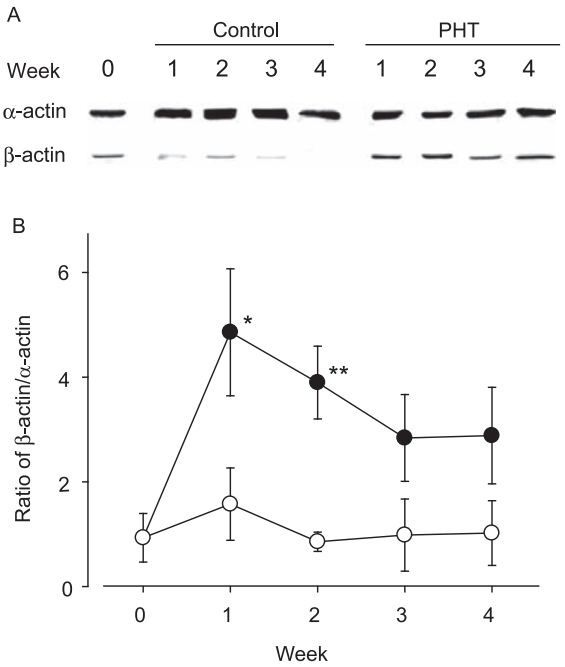

Fig. 3. Effect of hypoxia on expression ratio of β-actin to α-actin protein. A: One of the typical traces of Western blots. B: Quantitation of smooth muscle phenotype by indicating β-actin/α-actin ratio (n = 4 – 6). All results are means ± S.E.M. *P<0.05 and **P<0.01: significantly different from normoxic arteries. Control, normal rat; PHT, pulmonary hypertensive rat.

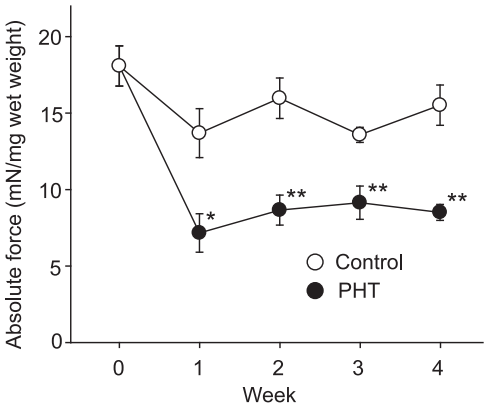

Fig. 4. Time-dependent changes in myogenic contractility of smooth muscle cells stimulated with 65.4 mM KCl in pulmonary arteries isolated from normoxic and hypoxic rats. Absolute force after 60 min and representative contraction of main pulmonary arteries from normoxic and hypoxic rats at 2 weeks (n = 10 – 105 from 4 – 18 rats). Results are means ± S.E.M. *P<0.05 and **P<0.01: significantly different from normoxic arteries. Control, normal rat; PHT, pulmonary hypertensive rat.

pulmonary arteries isolated from rats exposed to hypoxia for 1 – 4 weeks, causing a significantly increased β-actin/α-actin ratio after hypoxia for 1 – 2 weeks (Fig. 3).

Changes in contractile responses induced by chronic hypoxia

Figure 4 shows the maximal contractile responses to stimulation with 65.4 mM KCl in pulmonary arterial rings isolated from normoxic and hypoxic rats (1 – 4 weeks of exposure to hypoxia). The absolute force of the high K^+–induced contraction did not change in rings from normoxic rats over 4 weeks. In contrast, high K^+–induced contractility was significantly impaired at 1 week of exposure to hypoxia, and this impairment was maintained for 4 weeks.

Figure 5 indicates spontaneous changes in the basal active tonus of isolated pulmonary arterial rings. The basal active tonus was determined by adding 100 μM papaverine at the end of the experiments. In control pulmonary arteries isolated from normoxic rats at 1 week, the basal active tonus gradually decreased and a low steady state level was maintained within 30 min of starting the force measurement (Fig. 5A). The basal active tonus at 60 min after starting the force measurement was around 1.5 – 2 mN/mg wet weight. In contrast, the spontaneous basal active tonus in pulmonary arterial rings isolated from hypoxic rats gradually increased and reached a high steady state within 30 – 60 min after starting the measurements (Fig. 5B). The absolute force of basal active tonus at 60 min from the start of the experiment was significantly increased in arteries isolated from rats exposed to hypoxia for 1 – 3 weeks, but gradually decreased over time and completely disappeared in at 4 weeks of hypoxia (Fig. 5C).

Figure 5D shows the effects of various inhibitors on the basal active tonus of pulmonary arteries isolated from hypoxic rats to characterize the contractile mechanism. An ET_A blocker (CI-1034, 1 μM) and a rho-kinase inhibitor (Y27632, 10 μM) each decreased the basal active tonus by 25% – 30% within 60 min in pulmonary arteries isolated from rats exposed to hypoxia for 2 weeks. The L-type Ca^{2+} channel antagonist nicardipine (1 μM) also inhibited the basal tonus by approximately 60%.

Increase of ET-1 production in pulmonary hypertensive (hypoxic) lung

We measured the ET-1 content in lung tissues isolated from normoxic and hypoxic rats (Fig. 6). The lung tissue ET-1 content trended upward at 1 week of exposure to hypoxia, reached a maximum at 2 weeks, and then gradually decreased at 3 – 4 weeks.

Discussion

The present study found that the media of the

may become established during this period. This stage might be the most critical in the pathogenesis of PH and thereby early detection of this phase should be useful from a therapeutic viewpoint. We speculate that chronic proliferative activity is involved in the thickening of the media wall and pulmonary arterial stenosis. Hypoxia could act on fibroblasts and endothelial cells to trigger PASMC proliferation (29). These adjacent cells with PASMCs can release many growth factors for PASMC proliferation such as PDGF, FGF-2, and EGF (30 – 32). Most recently, it has been clarified that hypoxia can accelerate the proliferative activity of these growth factors via hypoxia-inducible factors-1 (HIF-1) (33). Further detailed pathophysiological examinations are required to clarify the relationship between proliferation of PASMCs and mitogenic factors for PASMCs in a chronic hypoxia–induced PH.

The numbers of mature PASMCs are increased in patients with primary PH (34); and in neonatal calves exposed to hypoxia for periods ranging from 2 – 16 days, PASMC are at their most proliferative at 8 days and vinculin-negative immature PASMCs particularly flourish among them (35). Our data support these findings and suggest that synthetic-type PASMCs are important in the development of PH. PASMC contractile ability was impaired to keep a steady level, although proliferative ability and the synthetic-type PASMC population was maximum at 1 week and then gradually decreased to maintain a low level throughout the experimental period. These results indicate the possibility that not only phenotypic change in PASMCs but also morphological factors may be related to inhibition of contractile ability. PASMC orientation, hyperplastic adventitia, and fibrosis may affect the contractile ability of the pulmonary artery (36).

Endothelin-1 is regarded as being potently active in the pathogenesis of PH (9 – 11). We examined ET-1 production in lung tissues containing pulmonary microvasculature and found that hypoxia increased ET-1 production after 1 – 4 weeks. The basal static contraction in the hypoxic artery was partially but significantly reduced by CI-1034, an ET_A-receptor blocker. These results suggest that chronic hypoxia increases lung ET-1 production, which affects ET_A receptors in PASMC, and causes continuous pulmonary vasoconstriction together with cell proliferation (9, 12, 37). However, ET-1–induced contraction might be mediated not only by a cellular Ca^{2+} increase but also by Ca^{2+} sensitization of contractile proteins in vascular smooth muscle (38, 39), and this pathway is mediated by a rhoA-dependent process (40). We found here that basal active tonus was partly but significantly inhibited by Y-27632, a potent rho-kinase inhibitor. In intrapulmonary arteries from chronically hypoxic rats, ET-1 induced Ca^{2+}-independent contractions mediated by rho kinase and tyrosine kinase but not PKC (21).

Sustained basal active vasoconstriction ceased at 4 weeks of hypoxia, whereas the high ET-1 level in the lung persisted, indicating that some intrinsic negative regulatory system is involved in hypoxia-induced increment of basal active tonus. Further investigations are necessary to clarify this point. In addition, the ET_A-blocker CI-1034 only partially blocked this static vasoconstriction. At least two mechanisms can account for the ET-1–independent contractile mechanism. One is that hypoxia directly induces pulmonary vasoconstriction via PASMC membrane depolarization. For example, PASMCs are continuously depolarized and accordingly cytosolic Ca^{2+} levels increase (41). Ito et al. found that increments of the basal active tonus of pulmonary artery in monocrotaline-induced pulmonary hypertensive rat are associated with membrane depolarization (42, 43). Although the depolarization mechanism is not fully understood, Cl^--channel activation can partly explain it (24, 44). We found here that the Ca^{2+}-channel antagonist nicardipine significantly inhibited the basal active tension, supporting this notion. It has been also reported that HIF-1 leads to decreased Kv-channel expression, which in turn causes subsequent membrane depolarization (12). In addition, store-operated Ca^{2+} channels possibly regulated by TRPc may also be related to elevation of basal Ca^{2+} level (45, 46).

Another mechanism is that other receptors associated with signaling molecules such as angiotensin-II and serotonin contribute to the induction of basal active tonus. Angiotensin-II and serotonin are not only vasoconstrictors but also cell proliferation activators, and their levels are increased by chronic hypoxia (47, 48).

In conclusion, the present study showed that chronic hypoxia causes an increase in the number of synthetic-type PASMCs, which results in pulmonary vascular remodeling indicated by cell proliferation and media wall thickening. Moreover, the sustained proliferative activity of PASMCs maintains the thickened media. On another front, the proportion of contractile-type PASMCs is relatively decreased, thus lowering the maximal contraction of the pulmonary artery. These phenomena might be associated with ET-1, which is a potent vasoconstrictor and cell proliferation activator.

Acknowledgments

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