Pyk2 aggravates hypoxia-induced pulmonary hypertension by activating HIF-1α

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Pyk2 aggravates hypoxia-induced pulmonary hypertension by activating HIF-1α. Am J Physiol Heart Circ Physiol 308: H951–H959, 2015. First published February 6, 2015; doi:10.1152/ajpheart.00770.2014.—Pulmonary arterial hypertension (PAH) is a refractory disease characterized by uncontrolled vascular remodeling and elevated pulmonary arterial pressure. Although synthetic inhibitors of some tyrosine kinases have been used to treat PAH, their therapeutic efficacies and safety remain controversial. Thus, the establishment of novel therapeutic targets based on the molecular pathogenesis underlying PAH is a clinically urgent issue. In the present study, we demonstrated that proline-rich tyrosine kinase 2 (Pyk2), a nonreceptor type protein tyrosine kinase, plays a crucial role in the pathogenesis of pulmonary hypertension (PH) using an animal model of hypoxia-induced PH. Resistance to hypoxia-induced PH was markedly higher in Pyk2-deficient mice than in wild-type mice. Pathological investigations revealed that medial thickening of the pulmonary arterioles, which is a characteristic of hypoxia-induced PH, was absent in Pyk2-deficient mice, suggesting that Pyk2 is involved in the hypoxia-induced aberrant proliferation of vascular smooth muscle cells in hypoxia-induced PH. In vitro experiments using human pulmonary smooth muscle cells showed that hypoxic stress increased the proliferation and migration of cells in a Pyk2-dependent manner. We also demonstrated that Pyk2 plays a crucial role in ROS generation during hypoxic stress and that this Pyk2-dependent generation of ROS is necessary for the activation of hypoxia-inducible factor-1α, a key molecule in the pathogenesis of hypoxia-induced PH. In summary, the results of the present study reveal that Pyk2 plays an important role in the pathogenesis of hypoxia-induced PH. Therefore, Pyk2 may represent a promising therapeutic target for PAH in a clinical setting.

PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive disorder characterized by sustained elevations in pulmonary arterial pressure and increased pulmonary vascular resistance. Despite advances in pharmacotherapeutics using prostanoids, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors, the prognosis of PAH remains poor, with a 5-yr survival rate of <70% (4).

Molecular targets need to be identified for the treatment of PAH on the basis of its pathogenesis to overcome therapeutic difficulties. Recent studies have demonstrated that the pathogenesis of PAH is closely associated with the proliferation, transformation, inflammatory responses, and mobilization of vascular smooth muscle cells (SMCs) (28). Since these biological processes are regulated by multiple cellular signal transduction pathways, some synthetic tyrosine kinase inhibitors may be promising drugs for the treatment of PAH. Dasatinib, a synthetic Src kinase inhibitor, and imatinib, a selective tyrosine kinase inhibitor, have been used to treat PAH. However, their curative effects and safety remain controversial (10, 20, 24). Thus, alternative therapeutic targets that are engaged in cellular signaling pathways need to be identified for the treatment of PAH. Proline-rich tyrosine kinase 2 (Pyk2) is a nonreceptor type, Ca2+-dependent tyrosine kinase that is involved in cell proliferation, differentiation, motility, energy metabolism, and stress responses (1, 3, 26). It is known to be activated by various extracellular stimuli, including hypoxia, which is one of the most probable causal factors of PAH. Pyk2 also plays pivotal roles in vascular remodeling. It has been shown to promote platelet-dependent growth factor-dependent proliferation of vascular SMCs, resulting in aberrant vascular remodeling, which is characterized by thickening of the wall (22). Although this vascular remodeling has been known to account for a part of the pathological process of PAH (8, 23, 27), the roles of Pyk2 in the cytokine-dependent vascular remodeling that is associated with PAH have not yet been elucidated in detail.

In addition to the pathogenic roles of cytokine-induced vascular remodeling, ROS have been previously shown to be involved in the molecular mechanisms underlying PAH (33). Pyk2 has been reported to modulate intracellular ROS production by activating NADPH oxidase or by affecting mitochondrial Ca2+ metabolism (21a). However, how Pyk2-dependent ROS generation is involved in the progression of PAH remains unclear.

Chronic hypoxia (CH) is one of the main causative factors for PAH. Most hypoxia-related cellular events, including vascular remodeling, are governed by hypoxia-inducible factor (HIF)-1α, a master transcription factor that regulates the hypoxic stress-responsive gene. HIF-1α gene heterozygous mice (HIF-1α+/− mice) are resistant to hypoxia-induced pulmonary hypertension (PH) (30, 34). Hypoxia-induced cellular signaling pathways partly overlap with Pyk2-related pathways. However, to the best of our knowledge, the role of Pyk2 in the HIF-1α-dependent pathogenesis of PAH remains unknown and has not yet been examined experimentally.

Using Pyk2 knockout (KO) mice, we demonstrated that Pyk2 played an important role in the pathogenesis of PAH by functionally interacting with HIF-1α-related pathways.
MATERIALS AND METHODS

Mice and hypoxia experiments in vivo. Pyk2 KO C57BL/6 strain mice were generated as previously described (17). All animal experiments were approved by and conducted according to guidelines of the Animal Ethical Committee of Kyoto Prefectural University of Medicine. Male mice (9–12 wk old, n = 30) were kept under hypoxic conditions (10% O2) in a ventilated chamber (KYODO, Tokyo, Japan). These hypoxic conditions were transiently interrupted once a week for cleaning of cages, feeding of mice, and measurements of body weights.

Cell culture and hypoxia experiments in vitro. Human pulmonary arterial SMCs (HPASMCs; Lonza, Walkersville, CA) were cultured in SmG-M-2 medium (Lonza) supplemented with SmG-M-2 Single-Quots (Lonza) according to the manufacturer’s instructions. After four to six passages, cells were grown to 80–90% confluence and subsequently incubated under normoxic (21% O2-5% CO2) or hypoxic (1% O2-5% CO2) conditions at 37°C in a hypoxic chamber (Astec, Fukuoka, Japan).

Gene-silencing experiments in vitro. HPASMCs were transfected with validated small interfering (si)RNA (Invitrogen, Carlsbad, CA) for Pyk2 or its scrambled RNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. After being transfected, treated cells were subjected to hypoxia or normoxia as described above.

Physiological assessment of PH. A thoracotomy was performed under positive pressure ventilation and general anesthesia with isoflurane. Right ventricular (RV) systolic pressure (RVSP) and heart rate were measured by inserting a catheter directly into the RV. We only adopted waveforms that were maintained for 1 min. The lungs and heart were subsequently removed. The ratio of RV weight to left ventricular plus septal weight (Fulton’s index) was measured to evaluate RV hypertrophy (RVH).

Cell migration and proliferation assays. Cell migration was evaluated using a “scratch” assay as previously described (15). In brief, siRNA Pyk2- or scrambled siRNA-transfected HPASMCs were allowed to grow to 100% confluence, after which a part of the cell monolayer was scratched. The migration distance was measured automatically by JuLI Br (NanoEnTek, Pleasanton, CA). Wound confluence was graphed to quantitatively analyze the recovery of the wound surface. Cell proliferation was examined using a water-soluble tetrazolium salt (WST-1) assay (Roche, Basel, Switzerland). HPASMCs were inoculated (2,500 cells/well) into 96-well plates. After incubation for 48 h, the WST-1 reagent was added directly into each well to detect mitochondrial dehydrogenase enzymes. The absorbance of each well was measured after 4 h at 450 nm with a reference reading at 630 nm.

Immunohistochemical experiments of lung tissues. Whole lung tissues were fixed in 4% paraformaldehyde, processed for paraffin embedding, and sectioned to generate 4-μm-thick sections. These sections were deparaffinized, rehydrated, and subsequently subjected to a microwave treatment in 50 mM citrate buffer. After endogenous peroxidase was quenched with 0.3% H2O2 for 20 min, slides were blocked with 10% goat serum and PBS (Niturei, Tokyo, Japan). They were subsequently incubated overnight with anti-α-smooth muscle actin (α-SMA) rabbit polyclonal antibody (1:100 dilution, Abcam, Cambridge, MA), anti-Ki67 antibody (1:50 dilution, Abcam), or anti-CD31 antibody (1:100 dilution, Abcam) at 4°C followed by an incubation with horseradish peroxidase-labeled anti-rabbit IgG antibody for 1 h at room temperature. The reaction was quenched with 0.3% H2O2 for 20 min, slides were blocked with 10% goat serum and PBS, and sections were counterstained with hematoxylin. Elastica van Gieson (EVG) staining was performed as previously described (18). Medial wall thickness was also determined as previously described (18). Wheat germ agglutinin (WGA) staining was performed as previously described (13).

RNA isolation and real-time PCR. Total RNA was isolated from the lungs or from cultured HPASMCs using an RNA extraction kit and nucleic acid purification kit (TAKARA, Otsu, Japan) according to the manufacturer’s protocols. cDNAs were generated with a Prime Script RT reagent kit (TAKARA). Real-time quantitative PCR was performed in combination with the SYBR green assay (Invitrogen). An initial denaturation step was performed for 10 min at 95°C followed by 40 cycles of amplification (55°C for 16 s, 72°C for 16 s, and 94°C for 20 s). Gene expression was quantified using β-actin as an internal control. PCR products were used by electrophoresis on 1.5% agarose gels with ethidium bromide staining to confirm the products. The following primers were used: mouse venge, sense 5′-ACTGGACCCCTGGCTTACTCTG-3′ and antisense 5′-TCATCCTATGGGACTTCTG-3′; mouse glucut, sense 5′-GCTTCGTGCTCATCACAATGCT-3′ and antisense 5′-CTCCTCTCTCCGCCATCATCT-3′; and mouse β-actin, sense 5′-CCGTGAAAGATGACCCCCAGA-3′ and antisense 5′-AGAGGCTACAGGGACACGCA-3′

Subcellular fractionation. Whole lung tissue was homogenized in lysis buffer [20 mM HEPES (pH 7.4), 120 mM NaCl, 2 mM KCL, 2mM MgCl2, 0.1% Nonidet P-40, 5% glycerol, 1 mM DTT, proteinase inhibitor cocktail, and phosphatase inhibitor cocktail]. The lysate was then centrifuged at 12,000 g for 10 min at 4°C. The resulting supernatants and pellets were designated as the postnuclear fraction and nuclear fraction, respectively. Fractionation was verified by Western blot analysis with anti-tubulin (Thermo, Waltham, MA) and anti-histone H3 (Abcam) antibodies. The nuclear fraction was used for the evaluation of lung HIF-1α. Whole tissue/cell proteins were extracted with Laemmli’s buffer (0.0625 M Tris-HCl, 2% SDS, and 10% glycerol; pH 6.8) followed by thorough shearing of the released DNA. Protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, CA).

Immunoblot analysis. Protein concentrations were determined by a CD protein assay kit (Bio-Rad). After being reduced, protein samples from whole lungs were subjected to Western blot analysis with antibodies against Pyk2 (Abcam), Tyr-phosphorylated Pyk2 (Cell Signaling, Beverly, MA), α-tubulin (Thermo), and HIF-1α (Bethyl Laboratories, Montgomery, TX). Signal intensities were determined using Image J software (National Institutes of Health, Bethesda, MD).

Measurement of intracellular ROS accumulation. The generation of ROS in HPASMCs was determined using ROS detection reagent (Molecular Probes, Carlsbad, CA). In brief, HPASMCs were incubated in growth medium containing 10μM chloromethyl-2′,7′-dichlorofluorescein diacetate at 37°C for 1 h under hypoxic conditions (1% O2). The fluorescence intensity of the cells was quantified using a confocal microscope (Fluoview FV1000, Olympus, Tokyo, Japan) at an excitation wavelength of 495 nm and emission wavelength of 527 nm for dichlorofluorescein. Signal intensities were determined using ImageJ.

Oxidative stress assay. Oxidative stress was evaluated by protein carbonylation, which is a marker for oxidative protein damage, using an OxyBlot protein oxidation detection kit (Millipore, Bellerica, MA). 4,8-Dinitrophenylhydrazine (DNPH) solution was freshly prepared (32). Quantitative changes in protein carbonyl levels were determined by immunoblot analysis.

Statistical analysis. Quantitative results are shown as means ± SE. Significance was evaluated by Tukey’s multiple-comparison method with StatMate III software (ATMS, Tokyo, Japan). Significance was set at P < 0.05.

RESULTS

Measurements of RVSP, RVH, heart rate, hematocrit, and body weight. WT and Pyk2 KO mice were exposed to the 10% O2 condition for 6 wk. Transient losses in body weight were observed in both WT and Pyk2 KO mice in the initial 2 wk. No significant differences were observed in changes of body weight between these groups during the 6-wk protocol (data not shown). On the basis of changes in RVSP and RVH, we evaluated the sensitivity to hypoxia-induced PH in WT and Pyk2 KO mice. As shown in Fig. 1A, RVSP of hypoxia-treated WT mice was 1.9-fold higher than that of normoxic WT mice (51.3 ± 4.5 vs. 26.8 ± 3.4 mmHg, P < 0.001). In contrast, RVSP of Pyk2 KO mice was 1.9-fold higher than that of normoxic Pyk2 KO mice (51.3 ± 4.5 vs. 26.8 ± 3.4 mmHg, P < 0.001).
mice was significantly lower than that of WT mice under the hypoxic condition (51.3 ± 4.5 vs. 38.4 ± 5.9 mmHg, *P* < 0.001; Fig. 1, *A* and *B*). A significant increase was also observed in RV weights in WT mice exposed to hypoxia (0.25 ± 0.02 vs. 0.39 ± 0.03 g, *P* < 0.05; Fig. 1C). No significant difference was observed in heart rate between WT and Pyk2 KO mice (Fig. 1D).

These results indicated that Pyk2 KO mice were resistant to hypoxia-induced PH.

**Pathological changes in the lungs of CH-treated WT and Pyk2 KO mice.** To investigate the role of Pyk2 in the pathogenesis of hypoxia-induced PH, we subjected lung tissues from control and hypoxia-treated mice to hematoxylin and eosin (H&E) staining, Masson’s trichrome (MTC) staining, EVG staining, or immunohistochemical staining for antibodies against α-SMA, Ki67, and CD31, which are markers of SMCs, proliferating cells, and endothelial cells, respectively.

As shown in Fig. 2, with H&E staining and immunohistochemical staining for α-SMA, medial wall thickness was increased in CH-treated WT mice, whereas such an increase was not observed in Pyk2 KO mice subjected to the same treatment. Furthermore, we quantitatively evaluated changes in medial wall thickness by EVG staining. This histological change was significantly inhibited in hypoxia-treated Pyk2 KO mice (Fig. 2C).

The number of Ki67-positive cells appeared to be higher in hypoxia-treated WT mice than in control WT mice. However, increases in the number of Ki67-positive cells were significantly attenuated in hypoxia-treated Pyk2 KO mice (Fig. 2D).

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**Fig. 1.** Effects of hypoxia on pulmonary vascular functions in wild-type (WT) and proline-rich tyrosine kinase 2 (Pyk2) knockout (KO) mice. WT and Pyk2 KO mice were both divided into two groups: 1) exposed to normoxia (21% O2) for 6 wk (*n* = 15) or 2) exposed to hypoxia (10% O2) for 6 wk (*n* = 15). *A–E*: right ventricular (RV) systolic pressure (*A* and *B*), wall thickness of the RV/left ventricle (LV) + septum (*C*), heart rate (*D*), and hematocrit (*E*) in both groups. Data are shown as averages ± SE. *P* < 0.05 and ***P* < 0.001 vs. Pyk2 KO (Pyk2−/−) mice in the hypoxic condition; †††*P* < 0.001 vs. WT (Pyk2+/+) mice under normoxic condition. ns, Not significant.
Fig. 2. Hypoxia-induced changes in pulmonary arterioles, hearts, and cardiac myocytes. Hypoxia-induced pathological changes in pulmonary small vessels (<100 μm) were investigated immunohistochemically. A total of ~30 vessels were examined in each mouse. Lung and heart tissues were excised from mice, as described in Fig. 1, and then subjected to hematoxylin and eosin (H&E) staining (A, G, H), elastica van Gieson (EVG) staining (C), Masson trichrome (MTC) staining (F, I, and J), wheat germ agglutinin (WGA) staining (K), and immunohistochemical examinations with antibodies against α-SMA (B), Ki67 (D), and CD31 (E) followed by counterstaining with hematoxylin. Changes in medial wall thickness were quantitatively evaluated by EVG staining (C, right). Vascular cell proliferation was quantitatively evaluated by immunohistochemical staining for Ki67 (D, right). The size of myocytes in the RV was quantitatively evaluated by WGA staining (K, right). Data are shown as averages ± SE; n = 6 mice/group. *P < 0.05, ***P < 0.001 vs. Pyk2 KO (Pyk2⁺/-) mice under the hypoxic condition; †P < 0.01 and †††P < 0.001 vs. WT (Pyk2⁺⁺) mice under the normoxic condition.
In addition, no obvious histological changes were detected in endothelial or adventitial fibrosis in vessels of control and hypoxia-treated mice (Fig. 2, E and F).

In summary, these results suggest that Pyk2 is involved in the hypoxia-induced cell proliferation of SMCs, which led to pulmonary arterial constriction.

**Pathological changes in the hearts of CH-treated WT and Pyk2 KO mice.** Heart tissues from control and hypoxia-treated mice were also investigated by H&E staining, MTC staining, and WGA staining. As shown in Fig. 2, G–J, no significant differences were observed in RV fibrosis or inflammatory cell invasion between these mice. Next, we quantitatively evaluated changes in RV myocyte hypertrophy by WGA staining (Fig. 2K). The size of RV myocytes was increased in CH-treated WT mice, whereas such an increase was not observed in Pyk2 KO mice subjected to the same treatment (Fig. 2K, graph).

**Biochemical study of Pyk2 with hypoxia.** HIF-1α plays central roles in hypoxia-associated tissue remodeling, including the proliferation of SMCs (29). Thus, we determined whether Pyk2 was involved in the pathogenesis of PAH through its modulation of the HIF-1α-associated pathway. Immunoblot analysis revealed that CH significantly increased the autophosphorylation of Pyk2 and HIF-1α protein levels in the whole lungs of WT mice (Fig. 3, A–C). However, this increase in HIF-1α protein levels was suppressed in CH-treated Pyk2 KO mice (Fig. 3, B and C). As shown in Fig. 3C, hypoxia significantly increased HIF-1α protein levels in the lungs of WT mice (up to 1.6-fold of baseline levels), Pyk2(+/+) 6h 12h 36h 1w 6w Pyk2(-/-) 0 0 12h 36h A α-Tubulin Pyk2 P-Pyk2 hypoxia Pyk2(+/+) Pyk2(-/-) hypoxia 12h Pyk2(+/+) Pyk2(-/-) normoxia Pyk2(+/+) Pyk2(-/-) hypoxia 12h

Fig. 3. Hypoxia-induced activation of Pyk2 in the lungs. The lungs were excised from mice, as described in Fig. 1, at the indicated time of hypoxia. A, left: the autophosphorylation of Pyk2 was evaluated by immunoblot analysis with antibodies against phospho-Pyk2 (top), Pyk2 (middle), and α-tubulin (bottom) as a loading control. B, left: nuclear hypoxia-inducible factor (HIF)-1α (top) and protein staining (bottom) as a loading control. C, left: changes in HIF-1α protein levels in hypoxia-treated WT (n = 3) and pyk2 KO mice (n = 3). The graphs show ratios of the signals of phospho-Pyk2 to Pyk2 (A, right) or HIF-1α to total protein staining (B and C, right). D and E: RT-PCR data were assessed as the ratio of mRNA expression of each molecule to the mRNA expression of VEGF (D) and glucose transporter 1 (GLUT1) (E) between WT and Pyk2 KO mice (n = 6). Data are shown as means ± SE. **P < 0.01 and ***P < 0.001 vs. Pyk2 KO (Pyk2−/−) mice under the hypoxic condition; †P < 0.05, ††P < 0.05, and †††P < 0.001 vs. control WT (Pyk2+/+) mice under the normoxic condition.

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whereas such hypoxia-induced increases were not observed in the lungs of Pyk2 KO mice.

Hypoxia-induced gene expression of VEGF and GLUT1 was also lower in Pyk2 KO mice than in WT mice (Fig. 3, D and E). These results demonstrate that the induction of HIF-1α was mediated by a Pyk2-related pathway.

Molecular mechanisms underlying Pyk2-dependent hypoxic responses in HPASMCs. Since Pyk2-dependent hypoxic histological changes were exclusively observed in SMCs of pulmonary arterioles, we used HPASMCs to investigate molecular mechanisms in vitro. As shown in Fig. 4A, the autophosphorylation of Pyk2 was rapidly increased in response to hypoxic stimulation. Since the hypoxia-induced induction of HIF-1α depended on Pyk2 in vivo (Fig. 3, B and C), we compared the induction of HIF-1α between Pyk2 knockdown and control cells. Concurrent with the time-dependent increase in Pyk2 phosphorylation, HIF-1α protein levels also gradually increased in HPASMCs subjected to 1% O2 for 8 h (Fig. 4B). The involvement of Pyk2 in cell proliferation was assessed using the WST-1 assay. siRNA-mediated knockdown of Pyk2 inhibited the proliferation of HPASMCs under hypoxic conditions (Fig. 4C). Differences in cell migratory potentials were compared between control and Pyk2 knockdown cells under hypoxic conditions using the monolayer wound-healing assay. Control HPASMCs (scrambled siRNA) reached 50% and 80% sealing in 8 and 12 h, respectively, after wound scratching. Pyk2 knockdown HPASMCs showed 50% sealing after 12 h, indicating that Pyk2 affected motility under hypoxic conditions (Fig. 4, D–F). A significant difference in cell motility was observed after 4 h (Fig. 4F). These results show that cell migration and proliferation activity were lower in Pyk2 knockdown cells.

Functional interaction between the HIF-1α-dependent pathway and Pyk2. A wide variety of hypoxic responses are known to be transcriptionally regulated by HIF-1α (16). However, little is known about the functional interaction between Pyk2 and HIF-1α. Previous studies have indicated that the expression of HIF-1α is mediated by oxidative stress (35), which is also known to be closely related to Pyk2 (7, 25).

Thus, we compared the extension of hypoxia-induced oxidative stress between control and Pyk2 knockdown HPASMCs. ROS production in living cells was determined with fluorescent dichlorofluorescein. As expected, intracellular production of ROS in normal cells was increased 2.2-fold by hypoxic stress (Fig. 5A). However, the production of ROS in Pyk2 knock-
down cells was 58% of that in the parent cells under the same condition (Fig. 5A).

Although the expression of HIF-1α was increased in HPASMCs under the hypoxic condition, its induction was less in Pyk2 knockdown cells under the same condition. To address the role of Pyk2 in hypoxia-induced oxidative stress, we also evaluated oxidative protein damage (protein carbonylation) with 2,4-DNPH and its specific antibody. As shown in Fig. 5, B and C, protein carbonylation rapidly but transiently increased in hypoxia-treated control cells, whereas no apparent increase in this change was detected in hypoxia-treated Pyk2 knockdown cells. In summary, these results suggest that Pyk2 is required for hypoxia-induced oxidative stress, which upregulates HIF-1α. To obtain a better understanding of the relationship between the induction of HIF-1α and hypoxia-induced cellular oxidative stress, we compared time-dependent changes in the induction of HIF-1α and protein carbonylation between normal and Pyk2 knockdown cells (Fig. 5C). Transient increases in protein carbonylation (maximum point, 1 h of hypoxia) preceded the induction of HIF-1α (maximum point, 3 h of hypoxia), suggesting that hypoxia-induced oxidative stress may be a prerequisite for the induction of HIF-1α. On the other hand, protein carbonylation and ROS production levels were markedly lower in Pyk2 knockdown cells than in control cells. Therefore, these results indicate that Pyk2 affected the induction of HIF-1α by modulating the cellular production of ROS under hypoxic conditions.

To examine the involvement of ROS in the Pyk2-dependent induction of HIF-1α, we investigated the effects of N-acetylcysteine (NAC) on HIF-1α expression levels. As shown in Fig. 5D, hypoxia-induced HIF-1α levels were significantly diminished in HPASMCs in the presence of N-acetylcysteine. Since cellular HIF-1α levels were posttranslationally regulated, Pyk2-dependent oxidative stress was required to stabilize HIF-1α protein under hypoxic conditions.

**DISCUSSION**

In the present study, we demonstrated that Pyk2 played an important role in the pathogenesis of PAH. We also showed that Pyk2 KO mice were less susceptible to hypoxia-induced PAH and that Pyk2 affected the stability of HIF-1α by modulating cellular oxidative stress under the hypoxic condition. Pyk2 has been identified as a critical intracellular signaling molecule that integrates chemokines, growth factor receptor stimulators, and regulators of cell migration. Impaired migration of macrophages has been previously reported in Pyk2 KO mice (21). Since PH is a progressive disorder in pulmonary vascular systems, pulmonary vessels, lymphocytes, and mac-

Fig. 5. Effects of ROS on HIF-1α activation. Cellular ROS production was determined with 2’,7’-dichlorodihydrofluorescein. A. left: HPASMCs were treated with siRNA for Pyk2 or its scrambled RNA (control) and then incubated for 1 h under normoxic or hypoxic conditions. Immunofluorescence signals were quantified using ImageJ (n = 5, P < 0.001; A. right). B and C: cellular oxidative stress was evaluated based on protein carbonylation. Carbonylated proteins were detected by immunoblot analysis with 2,4-dinitrophenylhydrazine and its specific antibody. α-Tubulin was used as a loading control. C: Time-dependent changes in protein carbonylation and HIF-1α expression were evaluated by immunoblot analysis. An increase in protein carbonylation proceeded the increased expression of HIF-1α in scrambled RNA-transfected cells. D: the effects of N-acetylcysteine (NAC) on HIF-1α expression levels were assessed. Data are shown as means ± SE; n = 4 each. ***P < 0.001; †††P < 0.001 vs. scrambled siRNA control cells under the normoxic condition.
rothrophages play important roles (31). Using Pyk2 KO mice and HPASMCs, we demonstrated that the activation of Pyk2 enhanced the migration of PASMCs and remodeling of the pulmonary artery. The suppression of inflammation was previously unable to prevent the development of PAH; therefore, the suppression of Pyk2 as a drug target may be an alternative treatment for PAH.

Hypoxia itself has been shown to increase intracellular Ca\(^{2+}\) concentrations under hypoxic conditions, which, in turn, activates Pyk2 through an interaction with calmodulin kinase (5, 9). A recent study by O-Uchi (21a) revealed that Pyk2 activated the mitochondrial Ca\(^{2+}\) uniporter to facilitate mitochondrial Ca\(^{2+}\) intake, and this also induced the mitochondrial production of ROS. In addition the leakage of mitochondrial electrons, which reduces molecular O\(_2\) to generate the superoxide anion radical, was also increased under hypoxic conditions. Based on these findings, we speculated that the activation of Pyk2 was involved in hypoxia-induced pulmonary artery remodeling.

Regarding the functional interaction between HIF-1\(\alpha\) and hypoxia-induced oxidative stress, we demonstrated that cellular oxidative stress was a prerequisite for the induction of HIF-1\(\alpha\). The potential implication of HIF-1\(\alpha\) in the pathogenesis of hypoxia-induced PH has been extensively debated during the past decade. A previous study (19) has reported that HIF-1\(\alpha\) played an important role in hypoxia-induced cell proliferation in vitro. Using smooth muscle-specific conditional HIF-1\(\alpha\) KO mice (HIF-1\(\alpha\)-SMM-Cre mice), Ball et al. (2) also demonstrated that attenuated expression of HIF-1\(\alpha\) suppressed the progression of hypoxia-induced PH. However, HIF-1\(\alpha\) gene expression was not completely suppressed in these mice. The residual expression of HIF-1\(\alpha\) may not lead to the development of hypoxia-induced PH because hypoxia-induced PH has been previously shown to be facilitated in other smooth muscle-specific conditional HIF-1\(\alpha\) complete KO mice (SM22a-HIF-1\(\alpha^{-/-}\) mice) (12). These models indicate that HIF-1\(\alpha\) plays a significant but not essential role in vascular remodeling. Although a rational explanation has not yet been provided for this discrepancy, it is likely that the excessive induction of HIF-1\(\alpha\) at the cellular level is a critical factor responsible for the progression of hypoxia-induced PH. In this scenario, the pathophysiological implication of the Pyk2-dependent signaling pathway in hypoxia-induced PH may be a cellular regulatory system for HIF-1\(\alpha\). Pyk2 may be an adequate mediator of HIF-1\(\alpha\) for hypoxia-induced PH.

A transducible dominant negative inhibitor of Pyk2, TAT-mediated protein transduction of dominant negative COOH-terminal Pyk2 (TAT-Pyk2-CT), has recently been developed (36). It has been shown to block the migration of neutrophils in a lipopolysaccharide-induced acute lung injury model (6). Although the effects of the chronic pharmacological inhibition of Pyk2 need to be elucidated in more detail, Pyk2 will be a novel therapeutic target of PAH.

In conclusion, we demonstrated that Pyk2 KO mice were resistant to hypoxia-induced PH. An in vitro studies using HPASMCs also showed that hypoxic stress increased cell proliferation and migration in a Pyk2-dependent manner. Since the activation of Pyk2 induced the ROS-mediated activation of HIF-1\(\alpha\), it was found to be crucial in hypoxia-induced pulmonary vascular remodeling.

**Limitations.** No significant difference was observed in the respiratory exchange ratio between Pyk2-deficient and WT mice at the baseline level (data not shown). However, metabolic changes may have occurred in Pyk2-deficient mice after 6 wk of hypoxia. Further examination of the metabolism in the lungs will facilitate the elucidation of the mechanisms of Pyk2-associated PH.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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