**Summary**

Pulmonary arterial hypertension is a fatal disease caused by pulmonary arterial vasoconstriction and organic stenosis due to the proliferation of pulmonary smooth muscle cells and endothelial cells. Endothelial dysfunction, including impaired nitric oxide (NO) bioavailability, plays a crucial role in the pathogenesis of pulmonary hypertension, and endothelial nitric oxide synthase (eNOS) is an important modulator of pulmonary vasodilatation. Although senescence marker protein (SMP) 30 is known as an anti-aging protein, the role of SMP30 in pulmonary vessels is still unclear. In this study, we examined the role of SMP30 in pulmonary vasculature using SMP30-deficient mice.

We used female SMP30-deficient mice and wild-type littermate (WT) mice at the age of 12 to 18 weeks. The WT and SMP30-deficient mice were exposed to normoxia or hypoxia (10% oxygen for 4 weeks). In normoxia, the right ventricular systolic pressure (RVSP) was not different between the WT and SMP30-deficient mice, but in hypoxia, the RVSP was significantly higher in the SMP30-deficient mice compared to the WT mice ($P < 0.05$). The hypoxia-induced increases in right ventricular hypertrophy and medial smooth muscle area of the pulmonary artery were comparable between the WT and the SMP30-deficient mice. Western blotting showed that eNOS phosphorylation in lung tissue was reduced in the SMP30-deficient mice compared to the WT mice in normoxia. However, in hypoxic conditions, eNOS phosphorylation was reduced in both the WT and SMP30-deficient mice with no differences in Akt phosphorylation.

Our study demonstrated that SMP30 is involved in the development of hypoxia-induced pulmonary hypertension by impairment of eNOS activity.

**Key words:** Anti-aging protein, Nitric oxide synthase, Endothelial dysfunction
SMP30 on cardiac muscles. We have also reported that SMP30-deficient mice exhibited reduced endothelium-dependent vasodilation and eNOS bioavailability. However, the effect of SMP30 on pulmonary arterial pressure has not yet been clarified.

In this study, we examined the role of SMP30 in pulmonary vasculature using SMP30-deficient mice.

**Methods**

**Animal ethics statement:** SMP30-deficient mice (C57BL/6 background) were established as previously reported. We used female SMP30-deficient mice and wild-type (WT) C57BL/6 mice aged 12-14 weeks (body weight range: 19.7 to 32.5 g). Both mice were fed a normal diet (CLEA Rodent Diet CA-1, CLEA Japan Inc., Tokyo) and were housed with food and water ad libitum at room temperature under a 12 h:12 h light-dark cycle. The WT mice and the SMP30-deficient mice were exposed to normoxia or hypoxia (10% oxygen) for 4 weeks. This study was conducted in accordance with the guidelines for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). Our research protocol was approved by the Fukushima Medical University Animal Research Committee, and we made all efforts to minimize animal suffering. After the experiment, all mice were sacrificed by cervical dislocation.

**Measurement of right ventricular systolic pressure and ventricular weight:** Anesthesia was performed by intraperitoneal injection of tribromoethanol (0.25 mg/g body weight). A 1.2 F micromanometer catheter (Transonic Scisense Inc., London, ON, Canada) was inserted via the right jugular vein, and right ventricular pressure was measured and analyzed using LabScribe 3 software (IWORX, Dover, NH, USA). In order to evaluate right ventricular hypertrophy (RVH), the Fulton index was calculated as the weight ratio of the right ventricle (RV) to the left ventricle (LV) and septum (S).

**Histological analysis:** After measuring the RV pressure, the lungs were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned to 3 μm slices. The pulmonary artery (20-50 μm external diameter) was randomly chosen after Elastica-Masson (EM) staining (60-90 vessels per mouse). The medial wall area (the area between the internal and external lamina) was measured using Image J 1.48 (National Institutes of Health, Bethesda, MD, USA) and was divided by the vessel area (the area surrounded by the external lamina).

**Western blotting:** Western blotting was performed as described previously. The poly vinylidene di-fluoride (PVDF) membranes were incubated with a mouse monoclonal antibody to eNOS diluted 1:1000 for 1 hour at room temperature. Rabbit polyclonal antibody to phospho-ylated eNOS (Ser 1177, Cell Signaling Technology, Beverly, MA, USA), Akt (Cell Signaling Technology), and phosphorylated Akt (Ser473, Cell Signaling Technology) were diluted 1:500. Each membrane was then incubated with a secondary antibody diluted 1:10,000 for 45 minutes.

**Statistical analysis:** Data were expressed as mean ± SD, and statistical analysis was performed using one-way analysis of variance followed by Scheffe’s test. A value of $P < 0.05$ was considered statistically significant.

**Results**

**Effect of SMP30 deficiency on RVSP and RVH:** In normoxic conditions, right ventricular systolic pressure (RVSP) in the SMP30-deficient mice was comparable with that in the WT mice. In hypoxic conditions, however, the RVSP in the SMP30-deficient mice was significantly higher than that of the WT mice. (29.2 ± 2.2 versus 25.8 ± 1.8 mmHg, $P < 0.01$) (Figure 1A). The Fulton index of the SMP30-deficient mice was higher than that of the WT mice in normoxia, although there was no significant difference statistically (Figure 1B). Exposure to hypoxia caused RVH in both the WT mice and the SMP30-deficient mice. The Fulton index of the SMP30-deficient mice was comparable that of the WT mice in hypoxia...
Effect of SMP30 on pulmonary hypertension

Vessel morphometry in SMP30-deficient mice: Histo-
logical analysis demonstrated that the area of the pulmo-

nary artery smooth muscle layer in the SMP30-deficient
mice was not significantly different from that in the WT
mice in normoxia. Exposure to hypoxia induced an
increase in the area of the pulmonary artery smooth muscle
layer in both groups of mice. However, no significant dif-
ference was observed between the two groups (Figure 2).

Effect of SMP30 on Akt/eNOS pathway in lung tissue:
Western blotting revealed that phosphorylation of eNOS in
the lung tissue was decreased in the SMP30-deficient
mice compared to that in the WT mice in normoxia. In
hypoxia, total eNOS protein levels were upregulated,
whereas the ratio of phosphorylated eNOS to total eNOS
was decreased both in the WT mice and the SMP30 de-
icient mice. The ratio of phosphorylated eNOS to total
eNOS in the hypoxia-exposed SMP30-deficient mice was
similar to that of the WT mice in hypoxia. On the other
hand, SMP30 deficiency or hypoxia did not have a sig-
nificant effect on the phosphorylation status of Akt in this
study (Figure 3).
Discussion

In the current study, we showed that SMP30 deficiency enhanced RVSP elevation by hypoxia in mice. Pulmonary vasoconstriction due to the decreased eNOS bioavailability contributed to the RVSP elevation, although thickening of the medial muscle layers of the pulmonary artery was not observed in the SMP30-deficient mice.

It was originally reported that SMP30 deficiency leads to a senile lung, which is characterized by peripheral airspace enlargement without alveolar destruction. However, structural changes, at least sufficient to increase RVSP, were not found in the lungs of the SMP30-deficient mice in the present study. Therefore, it was unlikely that the destruction of the pulmonary vascular bed caused pulmonary hypertension.

Thickening of the pulmonary artery smooth muscle layer due to proliferation of apoptosis-resistant smooth muscle cells or endothelial cells is a common finding in patients with pulmonary hypertension. The lungs of the SMP30-deficient mice in the current study did not show a significant increase of medial smooth muscle area in either normoxia or hypoxia. Although SMP30 has an anti-apoptosis effect, further study is needed to clarify the survival of pulmonary arterial smooth muscle cells in hypoxia-exposed SMP30-deficient mice. There was no difference in the Fulton index between WT and SMP30-deficient mice after hypoxia in this study. A possible reason for this discrepancy between RVSP and right ventricular hypertrophy was that a longer duration of high RVSP in response to hypoxic exposure might be necessary to cause significant differences in the right ventricular weight.

Impairment of eNOS phosphorylation due to SMP30 deficiency was observed in the aorta or the coronary artery in our previous reports. Therefore, it is believed that the difference in RVSP elevation without a difference in medial wall thickening between WT and SMP30-deficient mice in hypoxia might be due to the pulmonary artery vasospasm by the impaired eNOS phosphorylation in the SMP30-deficient mice. The detailed mechanism for the increased RVSP in SMP30-deficient mice without medial wall thickening of the pulmonary arteries should be further clarified. However, the eNOS phosphorylation level in the lung tissue of the SMP30-deficient mice was comparable with that of the WT mice under hypoxic conditions, whereas SMP deficiency caused a significant decrease in eNOS phosphorylation under normoxia. This may be because the effect of SMP30 deficiency on eNOS phosphorylation was small compared to the effect of hypoxia.

Generally, it is believed that eNOS phosphorylation is dependent mainly on Akt activity. However, Akt activity was not decreased by SMP30 deficiency or hypoxia, whereas eNOS phosphorylation was decreased in these conditions. This result suggests that Akt-independent eNOS phosphorylation may be involved in SMP30-deficient mice.

Recently, it has been reported that the reduction of Klotho protein, which is an anti-aging protein, is involved in the reduction of NOS of the pulmonary artery in monocrotaline-induced pulmonary hypertension rats. These results, including ours, suggest that anti-aging proteins regulate eNOS bioavailability, and affect the pathogenesis of pulmonary hypertension.

Conclusion

We demonstrated that SMP30 is associated with the pathophysiology of pulmonary hypertension in mice. Impairment of eNOS phosphorylation was involved in hypoxia-induced pulmonary hypertension in SMP30-deficient mice.

Disclosure

Conflicts of interest: Koichi Sugimoto and Tetsuro Yokokawa belong to an endowed department sponsored by Acterion Pharmaceuticals Japan. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. All other authors declare that no competing interest exists.