Research Article

Pulmonary Circulation

Gut microbiota modification suppresses the development of pulmonary arterial hypertension in an SU5416/hypoxia rat model

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Abstract

The pathogenesis of pulmonary arterial hypertension is closely associated with dysregulated inflammation. Recently, abnormal alterations in gut microbiome composition and function were reported in a pulmonary arterial hypertension experimental animal model. However, it remains unclear whether these alterations are a result or the cause of pulmonary arterial hypertension. The purpose of this study was to investigate whether alterations in the gut microbiome affected the hemodynamics in SU5416/hypoxia rats. We used the SU5416/hypoxia rat model in our study. SU5416/hypoxia rats were treated with a single SU5416 injection (30 mg/kg) and a three-week hypoxia exposure (10% O_2). Three SU5416/hypoxia rats were treated with a combination of four antibiotics (SU5416/hypoxia + ABx group) for four weeks. Another group was exposed to hypoxia (10% O_2) without the SU5416 treatment, and control rats received no treatment. Fecal samples were collected from each animal, and the gut microbiota composition was analyzed by 16S rRNA sequencing. The antibiotic treatment significantly suppressed the vascular remodeling, right ventricular hypertrophy, and increase in the right ventricular systolic pressure in SU5416/hypoxia rats. 16S rRNA sequencing analysis revealed gut microbiota modification in SU5416/hypoxia + ABx group. The Firmicutes-to-Bacteroidetes ratio in SU5416/hypoxia rats was significantly higher than that in control and hypoxia rats. Compared with the control microbiota, 14 bacterial genera, including *Bacteroides* and *Akkermansia*, increased, whereas seven bacteria, including *Rothia* and *Prevotellaceae*, decreased in abundance in SU5416/hypoxia rats. Antibiotic-induced modification of the gut microbiota suppresses the development of pulmonary arterial hypertension. Dysbiosis may play a causal role in the development and progression of pulmonary arterial hypertension.

Keywords

vascular remodeling, dysbiosis, pulmonary hypertension experimental, pathogenesis, inflammation

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Introduction

Pulmonary arterial hypertension (PAH) is a progressive cardiovascular disease characterized by elevated pulmonary arterial pressure, leading to right heart failure.[1] PAH

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prognosis is unfavorable, with a median survival of <3 years during the natural clinical disease course.[2] Although advanced diagnostic strategies and selective pulmonary vasodilators have contributed to improved PAH prognosis,[3,4] PAH is still incurable and demands life-long treatments.[5,6] Additionally, patients who have severe PAH and do not respond to maximal medical therapy require lung transplantation.[5,6]

Pulmonary vascular remodeling is a pathological feature observed in the pulmonary arteries in humans and experimental PAH models.[1,7,8] This remodeling causes obstruction of the pulmonary arteries and formation of complex vascular lesions, including plexiform-like, dilatation, or onion-skin lesions.[1,7] Furthermore, smooth muscle cells (SMCs) and endothelial cells (ECs) abnormally accumulate in remodeled pulmonary arteries.[1,9,10] The accumulation of SMCs and ECs involves mutated or dysfunctional transforming growth factor-β or bone morphogenic protein, and abnormal signal transduction.[1,9] Abnormal inflammation also plays a role in the progression of the vascular remodeling.[1,11] Inflammatory cells, including macrophages, T- and B-lymphocytes, dendritic cells, and natural killer cells are observed around remodeled vascular PAH lesions.[11] Elevated serum levels of inflammatory cytokines, such as interleukin-6 (IL-6) and tissue necrosis factor-α (TNF-α), are associated with poor PAH prognosis.[12,13]

The role of inflammation in the development of PAH has been investigated in experimental animal models. In the SU5416/hypoxia (Su/Hx) model, PAH is induced by chronic hypoxia exposure and SU5416, which is an inhibitor of the vascular endothelial growth factor receptor. This is a widely used representative animal model for PAH.[8,14] Chronically elevated pulmonary arterial pressure[15] and vascular remodeling are observed in Su/Hx rats,[8] similar to the observations in humans.[8] In a Su/Hx model of athymic rats lacking T cells, more severe pulmonary hypertension and vascular remodeling have been observed than in rats with normal immunity.[16] Therefore, abnormal inflammation and regulatory T cell dysfunction[16] are associated with the development of PAH.[1,11]

Microbiota includes the entire microbial community that populates a specific organ,[17] and the collective microbiota genome is called the "microbiome".[17,18] Recent technological advances have contributed to the understanding of microbiota and microbiome[19] and have revealed the relationship between "dysbiosis," which is abnormal microbiome composition and function,[17,18] and the development of cardiovascular diseases, such as atherosclerosis, systemic hypertension, and heart failure.[17,20,21] However, the involvement of dysbiosis in PAH development remains unclear.[22] Callejo and colleagues have recently reported alterations in gut microbiota composition and function in the Su/Hx rat model.[23] However, whether these alterations are a cause or the result of PAH is still unknown.

In this study, we investigated whether alterations in the gut microbiome affected the hemodynamics in Su/Hx rats. Accordingly, we modified the gut microbiota of Su/Hx rats through an antibiotic treatment and analyzed the differential microbiota pattern between the treated and untreated animals. Our results demonstrate that modifying the gut microbiota in Su/Hx rats suppresses the vascular remodeling and the deterioration of pulmonary hemodynamics.

Methods and materials

Animals

Five-week-old specific pathogen-free male Sprague Dawley rats were purchased from Japan CLEA Co. Ltd (Tokyo, Japan). The animals were kept in autoclaved cages ($22.5 \times 33.8 \times 14.0$ cm) with bedding at 24 °C under a 12-h light/dark cycle in the animal experimental facility of Chiba University. All rats had free access to food and sterilized water. General animal food (type MF) for rats was purchased from Oriental Yeast. Co., Ltd. Cages, bedding, drinking water, and food were replaced three times a week. Animals were randomly divided into four groups as follows: untreated rats (control group); rats exposed to only hypoxia for three weeks without SU5416 injection (Hx group); rats treated with SU5416 and exposed to hypoxia (Su/Hx group); and Su/Hx rats treated with antibiotics (Su/Hx + ABx group; Fig. 1).

Experimental PAH model

All rats were kept in normoxia for a week before the experiments for acclimation to the experimental room. Su/Hx rats were treated as previously reported.[8,24] Briefly, rats received

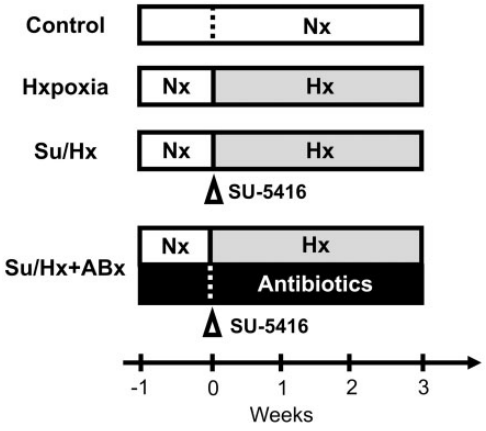

Fig. 1. Study protocol. One week before the beginning of the study, all rats were acclimatized in normoxic (Nx) conditions. The control group remained in Nx conditions. A second group of rats was exposed to hypoxia (10% O_2) for three weeks but did not receive SU5416 injection. A third group of rats received a subcutaneous injection of SU5416 (30 mg/kg) followed by exposure to hypoxia (10% O_2) for three weeks (Su/Hx group). A fourth group of rats received antibiotic treatment one week before and four weeks after receiving the SU5416 injection and exposure to hypoxia (Su/Hx + Abx group).

a single subcutaneous injection of 30 mg/kg SU5416 (R&D Systems, Minneapolis, MN) and then kept in ventilated hypoxic chambers (10% O_2, normobaric) for three weeks (Fig. 1).

Antibiotic treatment of Su/Hx rats

To modify intestinal bacteria composition, Su/Hx rats were treated with a cocktail of antibiotics (Fig. 1). The antibiotic cocktail contained 1 g/L ampicillin (Sigma), 500 mg/L vancomycin (Wako, Osaka, Japan), 1 g/L neomycin (Wako), and 1 g/L metronidazole (Wako), according to a previous report.[25] Antibiotics were dissolved in sterilized drinking water. The drinking water and food were replaced with fresh water and food three times a week. Antibiotic administration (pretreatment) was started one week before SU5416 treatment and continued until the end of the study period.

Hemodynamics measurements

Hemodynamics were evaluated by right-sided heart catheterization (RHC), as described in our previous report.[24] Briefly, rats were intraperitoneally anesthetized with 0.3 mg/kg medetomidine, 4 mg/kg midazolam, and 5 mg/kg butorphanol tartrate before RHC. A polyethylene catheter (internal diameter: 0.5 mm) was inserted through the right jugular vein and into the right ventricle (RV) to measure the right ventricular systolic pressure (RVSP). The catheter was connected to a transducer (LT0380/D; AD Instruments, Sydney, Australia). The signals were amplified with a Bio Amp (FE132; AD Instruments) and recorded using a PowerLab 8/35 computer system (AD Instruments). Wave data were analyzed using the Chart software (AD instrument). Rats were sacrificed by blood removal under anesthesia. The lungs and hearts were subsequently resected for pathological evaluations. The RV and left ventricle plus septum (LV + S) of each animal were weighed, and the RV/LV + S ratio, which is an indicator of RV hypertrophy,[26] was calculated.

Bacterial DNA isolation from fecal samples

Fecal samples were collected from each animal <1 h before starting hypoxia exposure at 0 weeks and the RHC at three weeks. Feces were directly collected from the anuses while being discharged and transferred to sterilized tubes by using sterilized needles. Each sample was frozen within 30 min of collection and stored at –80 °C until DNA isolation. Bacterial genomic DNA was extracted using a NucleoSpin Tissue DNA kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's data sheet.

16S rRNA analysis

A two-step PCR was performed for preparing the libraries of *16S rRNA* gene sequences. During the first step, the V3-V4 region of *16S RNA* gene was amplified using a 16S (V3-V4) Metagenomic Library Construction kit for NGS (Takara Bio) and primers 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3') according to the product datasheet. During the second step, the samples were indexed using a Nextera XT Index Kit (Illumina, San Diego, CA, USA) according to the datasheet. The prepared libraries were analyzed on a MiSeq instrument (Illumina) with 250-bp paired-end sequencing. Genome sequencing was performed at the Takara Bio Biochemical Center (Kusatsu, Shiga, Japan). Subsequent data processing was performed at the National Institutes of Biomedical Innovation, Health, and Nutrition. Clustering, assembly, chimeric evaluation, and operational taxonomic unit definition were performed using the QIIME pipeline (v 1.9.1), as previously described.[27] Taxonomy assignment was performed using the USEARCH algorithm at 97% similarity with SILVA 128 reference database. Principal coordinate analysis (PCoA) was performed using R package *vegan, ade4* based on Bray-Curtis distance matrix at the genus level, and clustering analysis was performed using the R package *vegan, stats* based on Bray-Curtis distance matrix at the genus level and by using the ward.D2 method (nboost = 10,000).

Quantification of bacterial abundance in fecal samples

The abundance of each bacterium in the fecal samples was estimated by quantitative PCR. The ZymoBIOMICS Spike-in Control I (Zymo Research, Irvine, CA, USA) was used as a bacterial standard. Twenty microliters of the standard sample contained 2×10^7 of *Imtechella halotolerans* and *Allobacillus halotolerans* cells. The standard samples were serially 10-fold diluted and used to generate a standard curve. The bacterial genomic DNA from the standard and fecal samples was extracted using a NucleoSpin Tissue DNA kit (Macherey-Nagel GmbH & Co. KG) according to the manufacturer's data sheet. Then, 2 μL of DNA samples, 14 μL of RT^2 SYBR Green Mastermix (Qiagen, Hilden, Germany), 1-μL forward primer (5′-ACT CCT ACG GGA GGC AGC AGT-3′), 1-μL reverse primer (5′-ATT ACC GCG GCT GCT GGC-3′), and 10 μL of DNase-free water were combined into a reaction mixture. PCR was performed using an ABI 7300 system (Applied Biosystems, Foster City, CA, USA). The PCR protocol was as follows: 10 min at 95 °C; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 32 s; followed by 15 s at 95 °C, 30 s at 60 °C, and the final ramp to 95 °C. Cycle thresholds were measured, and the abundance of each bacterium was calculated according to the standard curve.

Pathological examination

Resected lung tissues were fixed in 10% buffered formalin (Wako) for ≥ 48 h. The samples were embedded in paraffin

and sliced into 3-μm thick sections. Lung and heart tissues were stained with Elastica van Gieson staining. The pathological quantification of the pulmonary vessels was based on a previous report.[24] We identified all the pulmonary vessels in at least two slides per animal. The pulmonary vessels were scored by intimal occlusion severity as follows: 0, no neointima formation; 1, mild neointima formation and luminal narrowing (<50%); and 2, severe neointima formation and luminal occlusion (>50%). The histological findings were assessed by two investigators (T.J.S and H.S.) in a blinded manner, and final evaluations were based on their consensus.

Statistical analysis

Data were analyzed using GraphPad Prism (ver. 8.2.1, GraphPad Software Inc. San Diego, CA, USA). Continuous variables are described as means ± standard deviation unless otherwise stated. Differences between two groups were evaluated using the Student's *t*-test, and one-way ANOVA was used for assessing differences among multiple groups. The *p*-values were adjusted using the Bonferroni correction for multiple comparisons. $p < 0.05$ was considered significant.

Results

Modification of gut microbiota suppresses Su/Hx-induced RV hypertrophy and aggravation of hemodynamics

The RVSP values of control, Hx, Su/Hx, and Su/Hx + ABx groups were 22.6 ± 5.9, 50.0 ± 7.5, 57.7 ± 3.7, and 26.6 ± 6.0 mmHg, respectively (Fig. 2a). Su/Hx and Hx groups had significantly higher RVSP values than did the control group (control vs. Hx: $p < 0.0001$; control vs. Su/Hx: $p < 0.0001$). There was no significant difference between the RVSP values of Su/Hx + ABx and control groups ($p = 1.0$). The RVSP of Su/Hx + ABx group was significantly lower than those of Hx and Su/Hx groups (vs. Hx: $p = 0.0007$; vs. Su/Hx: $p < 0.0001$). The RV/LV + S ratios of control, Hx, Su/Hx, and Su/Hx + ABx groups were 0.20 ± 0.05, 0.33 ± 0.02, 0.55 ± 0.11, and 0.28 ± 0.04, respectively (Fig. 2b). Hx and Su/Hx rats had significantly higher RV/LV + S values than did the control group (control vs. Hx: $p = 0.02$, control vs. Su/Hx: $p < 0.0001$). The RVSP value of Su/Hx + ABx was significantly lower than that of the Su/Hx group ($p = 0.0004$), but did not differ from that of the control or Hx group (vs. control: $p = 0.6$, vs. Hx: $p = 1.0$).

Body weight

Fig. 2c shows the bodyweight data from each group. At 0 weeks, the body weights were as follows: control, 212.7 ± 5.3; Hx, 200.3 ± 5.9; Su/Hx, 210.5 ± 2.5; and Su/Hx + ABx, 128.0 ± 29.5 g. Su/Hx + ABx rats, which received one-week antibiotic pretreatment (Fig. 1) weighed significantly less than did the rats in the other groups (vs. control: $p < 0.0001$; vs. Hx: $p < 0.0001$; vs. Su/Hx: $p < 0.0001$). At three weeks, the body weights of the four groups were 390.0 ± 8.2, 254.0 ± 29.3, 314.0 ± 16.1, and 255.3 ± 18.1 g, respectively (Fig. 2c). The weights of Hx, Su/Hx, and Su/Hx + ABx rats were significantly lower than those of control rats (vs. Hx: $p < 0.0001$; vs. Su/Hx: $p = 0.0002$; vs. Su/Hx + ABx: $p < 0.0001$).

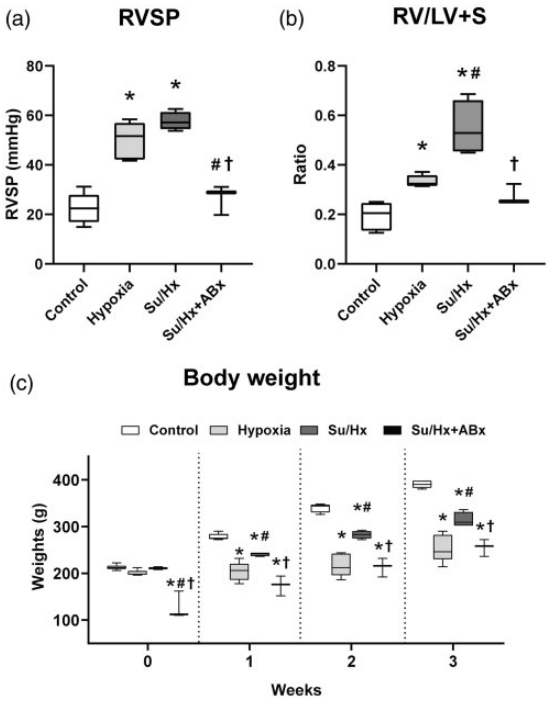

Fig. 2. Comparison of the hemodynamics and body weights among control, Su/Hx, and Su/Hx + Abx rats. (a) Right ventricular systolic pressure (RVSP). (b) The ratio of the right ventricle weight to left ventricle and septum weights (RV/LV + S). (c) Body weights of the four groups. Su/Hx: SU5416/hypoxia; Su/Hx + ABx: SU5416/hypoxia rats treated with antibiotics. *$p < 0.05$, vs. control; #$p < 0.05$, vs. Hx rats; †$p < 0.05$, vs. Su/Hx rats.

Gut microbiota modification suppresses Su/Hx-induced progression of vascular remodeling

The percent of obstructive vasculopathy (score 2) in control, Hx, Su/Hx, and Su/Hx + ABx groups were 1.1 ± 0.8, 6.8 ± 2.9, 40.1 ± 7.3, and 14.6 ± 6.3%, respectively. Su/Hx group had significantly more vessels with score 2 than the control or Hx group (vs. control: $p < 0.0001$; vs. Hx: $p < 0.0001$, Fig. 3). This difference was more pronounced than that observed between the Su/Hx + ABx group and the control or Hx group, indicating that the antibiotic pretreatment suppressed the development of obstructive vasculopathy ($p < 0.0001$) (Fig. 3).

Gut microbiota compositions

Fig. 4a and b show the gut microbiota compositions at the beginning and end of the study. The ratio of Firmicutes-to-Bacteroidetes (F/B) ratio, which is a biomarker of

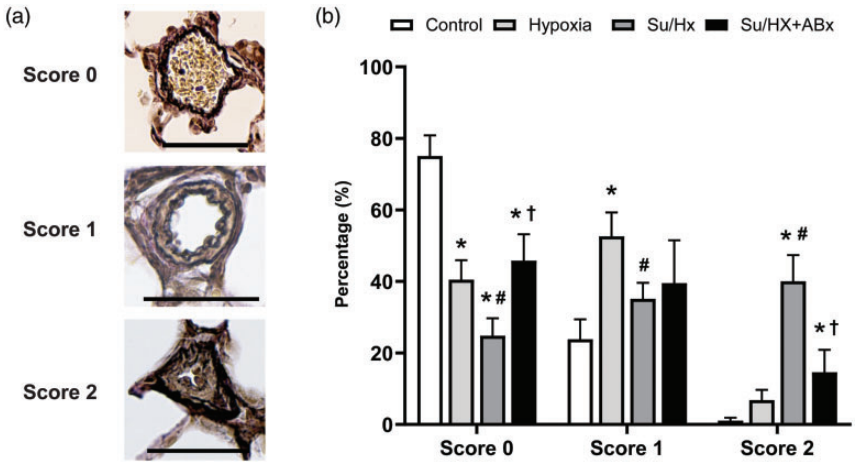

Fig. 3. Vascular remodeling in control, Su/Hx, and Su/Hx + ABx rats. (a) Representative images showing each grade of vascular remodeling. Scale bars show 50 μm. (b) The proportion of each grade of vascular remodeling among the three groups.
Su/Hx: SU5416/hypoxia; Su/Hx + ABx: SU5416/hypoxia rats treated with antibiotics.
*$p < 0.05$, vs. control; #$p < 0.05$, vs. Hx rats; †$p < 0.05$, vs. Su/Hx rats.

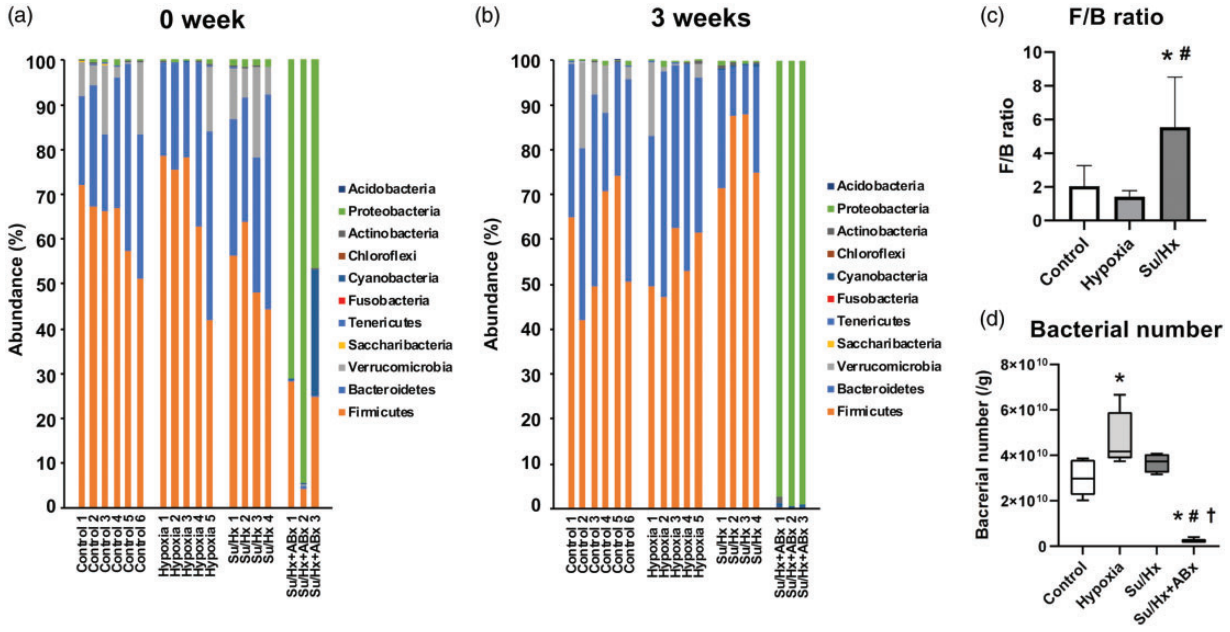

Fig. 4. Gut microbiota compositions. (a) The gut microbiota compositions at the beginning of the study. (b) The gut microbiota compositions after three weeks of hypoxic exposure. (c) The Firmicutes-to-Bacteroidetes (F/B) ratios in control, hypoxia, and Su/Hx groups at three weeks. (d) The bacterial numbers in fecal samples at three weeks.
Su/Hx: SU5416/hypoxia; Su/Hx + ABx: SU5416/hypoxia rats treated with the antibiotic cocktail.
*$p < 0.05$, vs. control; #$p < 0.05$, vs. Hx rats; †$p < 0.05$, vs. Su/Hx rats.

dysbiosis,[28] was significantly higher in Su/Hx rats than in the control and Hx groups (vs. control: $p = 0.02$, vs. Hx: $p = 0.01$, Fig. 4c) at three weeks, although there was no difference among the three groups at 0 weeks ($p = 0.3$). The abundance of Bacteroides was 0% in all the Su/Hx + ABx rats, and therefore, the F/B ratios in these rats were incalculable.

Antibiotic treatments modify gut microbiota composition in rats

Fig. 4d shows the total number of bacteria in the fecal samples from each group. The bacterial number in the Su/Hx + ABx group was significantly lower than those in the other groups (vs. control: $p = 0.002$; vs. Hx: $p < 0.0001$;

Su/Hx: $p < 0.0005$). Moreover, the gut microbiota composition in Su/Hx + ABx rats was different from those of the other groups (Fig. 4a and b). At the phylum level, the abundance of Proteobacteria in the Su/Hx + ABx group was $98.6 \pm 1.1\%$ at three weeks and was significantly higher than those in other groups (control: $0.5 \pm 0.5\%$, $p < 0.0001$; Hx: $0.5 \pm 0.5\%$, $p < 0.0001$; Su/Hx: $0.8 \pm 0.3\%$, $p < 0.0001$). At the genus level, *Shigella* accounted for $95.8 \pm 3.1\%$ of the gut microbiota in Su/Hx + ABx rats (Supplemental Fig.).

Gut microbiota composition differ between PAH and control rats

16S rRNA analysis revealed that Su/Hx rats had a microbiota composition distinct from that of control rats at three weeks (Fig. 5). PCoA, which is a metric analysis for visualizing the similarities and differences among communities,[19] revealed that control and Su/Hx groups formed different clusters (Fig. 5a). The results of dendrogram analysis also showed differences in gut microbiota composition between control and Su/Hx groups (Fig. 5b). Fig. 5c shows the microbiota compositions of the two groups at the phylum level. Firmicutes, Actinobacteria, and Cyanobacteria were significantly less abundant in the Su/Hx group than in the control group (Firmicutes: $p = 0.03$; Actinobacteria: $p = 0.03$; and Cyanobacteria: $p = 0.009$; Fig. 5c). The abundance of Bacteroidetes was also lower, although the difference was not significant ($p = 0.07$; Fig. 5c). Fig. 5d shows the differential bacterial composition pattern between control and Su/Hx groups at the genus level. The abundances of

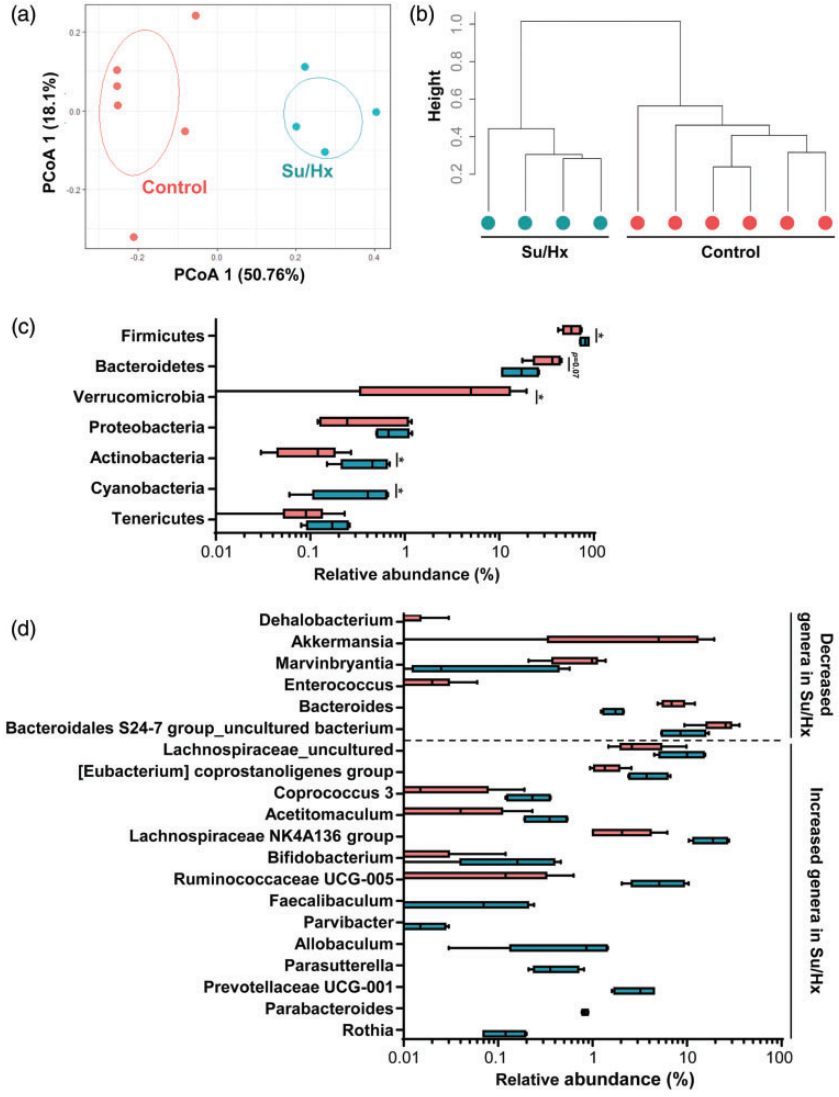

Fig. 5. Differences in gut microbiota between control and Su/Hx rats at three weeks. (a) Principal coordinates analysis (PCoA). (b) Dendrogram of the two groups. (c) The compositions of gut microbiota at the phylum level. *$p < 0.05$, vs. control. (d) Significantly increased and decreased genera in Su/Hx rats compared with those in the control group.
Su/Hx: SU5416/hypoxia.

six bacteria were significantly decreased in the Su/Hx group when compared with the control (*Dehalobacterium:* $p = 0.025$; *Akkermansia:* $p = 0.048$; *Marvinbryantia:* $p = 0.043$; *Enterococcus:* $p = 0.034$; *Bacteroides:* $p = 0.014$; and Bacteroidetes S24-7 group uncultured bacterium: $p = 0.043$). Conversely, the abundances of 14 bacteria were significantly increased in the Su/Hx group (Lachnospiraceae uncultured: $p = 0.043$; *Eubacterium coprostanoligenes* group: $p = 0.025$; *Coprococcus 3:* $p = 0.040$; *Acetitomaculum:* $p = 0.042$; Lachnospiraceae NK4A136 group: $p = 0.014$; *Bifidobacterium:* $p = 0.017$; Ruminococcaceae UCG-005: $p = 0.014$; *Faecalibaculum:* $p = 0.026$; *Parvibacter:* $p = 0.026$; *Allobaculum:* $p = 0.0057$; *Parasutterella:* $p = 0.0057$; Prevotellaceae UCG-001: $p = 0.0057$; *Parabacteroides:* $p = 0.0057$; and *Rothia:* $p = 0.0056$).

Discussion

In this study, we first explored the effects of altered gut microbiota in a PAH rat model. The antibiotic treatment modified the gut microbiota of Su/Hx rats, and the Su/Hx rats treated with antibiotics developed less vascular remodeling and PAH. The results suggested that gut microbiota is possibly related to the pathogenesis of PAH.

Recently, Callejo et al. have reported the altered composition of gut microbiota in Su/Hx rats as compared with control rats.[23] They also described that the F/B ratio was higher in Su/Hx rats than in control rats, consistent with our results. Several other studies have described the relationship between cardiovascular diseases and dysbiosis.[20,21,23,28] For example, Yang et al. have demonstrated that F/B ratios are higher in two different animal models of systemic hypertension than in control rats.[28] They have also shown that patients with systemic hypertension have distinct gut microbiota compositions from those of healthy control subjects.[28] Moreover, Emoto et al. have reported that alterations in gut microbiota can be related to the severity of coronary artery disease.[20]

The underlying cause of dysbiosis in PAH remains unclear. Hypoxia exposure reduces the oxygen pressure within the small intestine, which can induce alterations in gut microbiota.[29] In the current study, there was no difference in the F/B ratios between the control and Hx rats. The discrepancy suggests that other factors can be involved with dysbiosis in Su/Hx rats. There is no report describing that the effects of SU5416 on gut microbiota, although the possibility that SU5416 treatments may play a potential role in the dysbiosis cannot be excluded. Sympathetic nervous system is activated in Su/Hx rats.[30] Activated sympathetic nervous system can increase the permeability of the gut,[31] which may be related to dysbiosis in Su/Hx rats.[23] Heart failure patients have increased gut permeability and gut dysbiosis,[32,33] although it is still unclear whether dysbiosis is a cause of heart failure. Thus, the dysbiosis in Su/Hx rats may depend on several different systems. Our results suggest the causative role of dysbiosis in Su/Hx rats, but whether the dysbiosis is a cause or the result of PAH is open to discussion. The precise mechanisms of dysbiosis in PAH should be investigated in future studies.

We observed an imbalance in the gut microbiota composition of Su/Hx rats relative to that of the control rats both at the phylum and genus levels. *Bacteroides* and *Akkermansia*, which are related to the suppression of inflammation, were decreased in Su/Hx rats. *Bacteroides* are known to be responsible for the induction of regulatory T cells (Tregs) via polysaccharide A production.[34,35] Tregs suppress the excessive inflammatory response after vascular injuries, and their reduction may induce the development of PAH.[16,36] *Akkermansia* suppresses vascular inflammation and the development of atherosclerosis via suppression of the expressed inflammatory cytokines, such as TNF-α and IL-1β.[37,38] Kim et al. have recently reported that the abundances of *Bacteroides* and *Akkermansia* are decreased in PAH patients compared with those in control subjects.[39] These decreases might be related to the development of PAH both in humans and experimental animal models. Increased bacteria in Su/Hx rats, such as *Rothia* and *Prevotellaceae*, might be associated with the induction of inflammation. *Rothia dentocariosa* is commonly isolated from the oral cavity and is associated with TNF-α production and chronic inflammation in periodontal diseases.[40] Furthermore, *Rothia* is related to increased IL-8 levels and disease severity in patients with obstructive sleep apnea syndrome.[41] Increased *Prevotellaceae* levels are commonly observed in patients with new-onset untreated rheumatoid arthritis (RA),[42,43] and plays a causative role in the development of RA.[43] Thus, we think that imbalanced abundances of several different bacteria might be associated with the induction or suppression of inflammation, which might be related to the development of PAH.

Modification of the gut microbiota using antibiotics suppressed the progression of PAH in our study. Indeed, antibiotic treatments dramatically altered the gut microbiota composition in Su/Hx + ABx rats. *Shigella* accounted for > 95% of the gut microbiota in Su/Hx + ABx rats. It has been acknowledged that *Shigella* is not pathogenic to rats, mice, rabbits, or guinea pigs.[44,45] This bacterium can acquire resistance to antibiotics, including β-lactams, aminoglycosides, and fluoroquinolones, as a result of abused antibiotic treatments.[45,46] Thus, we think that microbial substitution occurred in Su/Hx + ABx rats.

Antibiotic treatments can suppress the development of cardiovascular diseases. Yang et al. have described that minocycline improves angiotensin II-induced systemic hypertension via restoring the gut microbiota.[28] Koeth et al. have reported that broad-spectrum antibiotic treatments suppress the progression of atherosclerosis induced in *Apoe* knockout mice.[47] Other gut microbiota disturbances may also affect cardiovascular diseases. Marques et al. have demonstrated that high-fiber diet changes the gut microbiota and suppresses the progression of systemic

hypertension and heart failure in mice with mineralocorticoid-induced hypertension.[48] These data support the notion that dysbiosis plays causal roles in the development of PAH and restoring the gut microbiota might offer an alternative strategy for the prevention of PAH.

In the current study, the body weights of Su/Hx + ABx rats were lower than those of the rats in the other groups. Gut microbiota play important roles in energy biogenesis and vitamin synthesis.[17] The lack or modification of gut microbiota can affect body weight. For example, mice that are housed under germ-free conditions and thus have not been exposed to any living bacterium weigh 40% less than those housed under regular conditions.[49] Treatments using multiple antibiotics can also induce weight losses in rats.[50] Finally, chronic hypoxia exposure can be a cause of weight loss in rats.[51] Thus, it seems that the weight loss in Su/Hx + ABx rats in our study might have resulted from the combined effects of antibiotic and hypoxia treatments.

The current study had some limitations. First, the body weights of rats in the three groups were different, which might have affected the observed changes in gut microbiota and in the progression of PAH. Second, the sample size of Su/Hx + ABx rats was small. Third, we did not analyze the function of the gut microbiota by metagenome analysis. Fourth, the therapeutic effect of antibiotic treatments for PAH was unclear under the design of the current study. The reason for this was the fact that the antibiotic treatment in this study was preventive and not therapeutic. The therapeutic effects of altering gut microbiota on PAH should be investigated in the future studies. Finally, our results were obtained only from animal experiments. Therefore, it is unclear whether our results are applicable to human patients with PAH. At this moment, we do not recommend long-term antibiotic treatment for these patients with PAH and the risk factors because such it would be too invasive. In future clinical studies, other interventions, such as probiotic treatments, should be considered. Despite these limitations, we believe that the results of this study indicate the important relationship between the pathogenesis of PAH and gut microbiota.

In conclusion, modification of gut microbiota with antibiotics suppresses the development of PAH. Dysbiosis may play a causal role in the development and progression of PAH, and modification of gut microbiota might be a new option for the prevention of PAH.

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Conflict of interest

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Ethical statements

All the experimental procedures involving animals were approved by Chiba University Instrumental Animal and Use Committee (approval numbers 29-231 and 30-446).

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Supplemental Material

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