High-Fat Diet Attenuates the Improvement of Hypoxia-induced Pulmonary Artery Hypertension in Mice During Reoxygenation.

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Research Article

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Abstract

Background: It has been widely recognized that metabolic disorder is associated with pulmonary hypertension. It is known that elevated pulmonary artery pressure induced by hypoxia in mice returns to normal pressure under reoxygenation. However, it is still unclear how metabolic disorder affects the reverse remodeling of pulmonary arteries. In this study, we investigated the effects of a high-fat diet (HFD) on the decrease of pulmonary artery pressure and reverse remodeling of pulmonary arteries in mice with hypoxia-induced pulmonary hypertension.

Methods: We used female C57/Bl6 mice aged 8 weeks. After the mice were exposed to hypoxia (10% oxygen for 4 weeks) for induction of pulmonary hypertension. Then, they were returned to normoxic conditions and randomized into a normal diet (ND) group and a HFD group. Both groups were fed their respective diets for 12 weeks.

Results: The right ventricular systolic pressure measured by a micro-manometer catheter and the Fulton index were significantly higher in the HFD group than in the ND group at 12 weeks after reoxygenation. The medial smooth muscle area was larger in the HFD group. Caspase 3 activity in lung tissue of the HFD group was decreased, and an apoptosis of pulmonary smooth muscle cells was suppressed after reoxygenation. Moreover, the expression levels of peroxisome proliferator-activated receptor-γ and apelin were lower in the HFD group than in the LFD group.

Conclusion: Our results suggest the metabolic disorder may suppress pulmonary artery reverse remodeling in mice with hypoxia-induced pulmonary hypertension under reoxygenation.

Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening disease leading to right heart failure. Until some twenty years ago, the 5-year survival rate of PAH patients was less than 50% [1]. Pathologically, PAH is characterized by pulmonary vasoconstriction and organic stenosis due to abnormal proliferation of pulmonary vascular cells [2, 3]. The mechanisms of these cancer-like proliferations of pulmonary vascular cells are not yet thoroughly understood.

Recent PAH-specific therapies using phosphodiesterase-5 inhibitors, endothelin receptor antagonists, prostacyclin, and guanyl cyclase stimulator, have markedly improved the prognosis of PAH patients [4]. Although the mechanisms are unknown, these vasodilators may indirectly suppress the development of pulmonary arterial remodeling. However, the mechanisms of pulmonary arterial reverse remodeling in the treated PAH patients are unclear.

Several studies have reported that metabolic disorder is associated with pulmonary hypertension [5–7]. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is well known as an important key molecule for metabolic syndrome. PPAR-γ also plays a crucial role in the pathogenesis of pulmonary hypertension [8–

10]. Alastalo et al. reported that PPAR-γ/β-catenin axis upregulates apelin that induces the apoptosis of pulmonary smooth muscle cells [10].

It is known that elevated pulmonary arterial pressure induced by hypoxia in mice returns to normal pressure under reoxygenation [11]. However, it is still unclear how metabolic disorder affects the reverse remodeling of pulmonary arteries. In the present study, we investigated the effect of a high-fat diet (HFD) on the decrease of pulmonary artery pressure and reverse remodeling of pulmonary arteries using mice with hypoxia-induced pulmonary hypertension.

Methods

Animals and ethics statement

We used littermate female C57BL/6 mice aged eight weeks (body weight range: 18.1–20.0 g). Mice were housed with food and water ad libitum at room temperature under a 12:12 light-dark cycle. The investigations conformed to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th Edition, 2011), and our research protocol was approved by the Fukushima Medical University Animal Research Committee. The study on animals was carried out in compliance with the ARRIVE guidelines. All efforts were made to minimize the suffering of the animals, all of which were sacrificed by cervical dislocation after the experiments.

Animal experiment

The experimental protocol has been shown in Fig. 1. Experiment 1: The mice were exposed to hypoxia (10% oxygen) or normoxia conditions for 4 weeks during which they were given normal chow. Thereafter, we evaluated right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH), and histological features. Experiment 2: The mice were exposed to hypoxia condition for 4 weeks during which they were given normal chow. Later, normoxia was resumed, and the mice were divided by a person who was blind to the investigation into the following two groups: the ND group mice were fed a normal diet (CLEA Rodent Diet CA-1, CLEA Japan Inc., Tokyo, Japan) and the HFD group mice were fed a high fat diet (High Fat diet 32, CLEA Japan Inc.) for 12 weeks. The oxygen concentration of hypoxia was determined according to the previous study [11]. The required sample size was estimated to be 10–20 per group based on the number within the range reported in previous studies [12, 13]. The used animal number was described in each figure legends. The total number of animals used was 63. Mice were carefully monitored daily during the animal study by evaluating the general appearance. When the mice showed the signs of significant clinical cardio-respiratory distress such as increased respiratory rates, reduced activity, piloerection, hunched posture, the mice were due to be euthanized by CO_2 exposure. However, no mice exhibited such findings or died before the scheduled termination. We set to begin to treat the mice at once in all the groups, and each group was allocated in each cage. Then, after we unified the cage locations, we randomly measured the RVSP to avoid the effect of order of animal and cages.

Measurements of right ventricular pressure and ventricular weight

Anesthesia was performed by intraperitoneal injection of Tribromoethanol (0.25 mg/g of body weight). A 1.2F micromanometer catheter (Transonic Scisense Inc., London, ON, Canada) was inserted from the right jugular vein, and RVSP was measured and analyzed by LabScribe3 software (IWORX, Dover, NH, USA) [14]. Post the measurement of RVSP, the mice were sacrificed by cervical dislocation; the heart and lungs were removed for the evaluation of RVH. The right ventricle (RV) was dissected from the left ventricle (LV), including the septum (S), and the RV/LV + S weight ratio (Fulton index) was calculated [14, 15]. Mice with severe bleeding or pneumothorax that affected hemodynamics during catheterization were excluded from the RVSP analysis.

Histological analysis

After measurement of RV pressure, the lungs were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned to 3 μm. After Elastica-Masson (EM) staining or immunostaining of α-smooth muscle actin (α-SMA) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), pulmonary arteries (external diameter of 20–50 μm) were randomly selected (60–90 vessels per individual mouse). The medial wall area (the area between the internal and external lamina) was measured using Image J 1.48 software (National Institutes of Health, Bethesda, MD, USA), and was divided by the vessel area (the area surrounded by the external lamina) [14, 15]. Furthermore, each vessel (external diameter < 20 μm) was classified as non-muscular, partially muscular, or fully muscular, as described in a previous report. The percentage of muscularized pulmonary vessels was determined by dividing the sum of partially and fully muscular vessels by the total number of vessels [14]. Measurements were performed blinded to mouse information.

Lipids profiles

Concentrations of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) in plasma were measured using ELISA kits according to the instruction at 24 weeks.

Measurements of mitochondrial ATP

To evaluate the mitochondrial function, we measured the mitochondrial adenosine triphosphate (ATP) in the lungs, as per a previous study [11]. Briefly, the removed lung was homogenized, and the mitochondria fraction was isolated by centrifugation. Thereafter, ATP production was measured using a commercially available kit (TOYO B-Net Co., Ltd. Tokyo, Japan) as per the manufacturer's instruction.

Western blotting

Western blotting was performed as described previously [16, 17]. The Poly Vinylidene Di-Fluoride membranes were incubated with a mouse monoclonal antibody to β-actin diluted 1:1000 for 1 hour at room temperature. Rabbit polyclonal antibody to active caspase-3 (Cell Signaling Technology, Beverly, MA, USA), PPAR-γ (Cell Signaling Technology) and Apelin (Bioss Inc, Boston, MA, USA), apelin (Bioss

Antibodies, Boston, MA, USA) were diluted 1:500. Each membrane was then incubated with a secondary antibody diluted 1:10000 for 45 minutes.

Apoptosis of pulmonary smooth muscle cells

To estimate the apoptosis of pulmonary smooth muscle cells, terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) assay (Promega, Madison, WI, USA) was performed according to the manufacturer's instructions. We randomly selected at least 10 fields in each specimen, and counted the nuclei in the medial smooth muscle layer. The results were expressed as percentages of the number of TUNEL-positive nuclei in the total number of nuclei [14].

Statistical analysis

Data were expressed as mean ± SD, and statistical analysis was performed using either Student's t-test or one-way ANOVA with Bonferroni's multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of hypoxia on RVSP and RVH

In experiment 1, we confirmed the effect of hypoxia exposure onto RVSP, RVH and medial thickening at 12 weeks. Both RVSP and Fulton index were elevated in response to hypoxia (Figs. 2A and B). The medial smooth muscle layer of the hypoxia-exposed mice was significantly greater than that of the non-hypoxia-exposed mice (Fig. 2C).

Effect of HFD on hypoxia-induced PAH mice during reoxygenation

Figure 3A shows that body weight in the HFD group was significantly heavier than that of in the ND group at 24 weeks in Experiment 2. glucose, LDL-C and HDL-C, but not TG, in the HFD group were significantly higher than those in the ND group (Fig. 3B). There was a significant difference in RVSP between the ND and HFD groups at 24 weeks; that in the ND group was almost recovered to normal level, whereas that in the HFD group was not (Fig. 3C). RV weight and Fulton index in the HFD group were larger than those in the ND group, whereas LV weight was comparable between the groups (Fig. 3D). Figure 3E shows the levels of mitochondrial ATP in the lung tissues. The mitochondrial ATP in the HFD group was higher than that in the ND group.

HFD delays reverse remodeling of pulmonary artery

In the ND group, the hypoxia-induced medial wall thickening of the pulmonary arteries almost normalized at 24 weeks, whereas it remained significant in the HFD group (Fig. 4A, upper panels). The proportion of fully muscular vessels in the HFD group was significantly higher than that in the ND group. The

percentages of partially muscular vessels were nearly equivalent between the two groups. (Fig. 4A, lower panel). Western blotting demonstrated that caspase-3 activity was decreased in the lung tissue of the HFD group (Fig. 4B). TUNEL staining shows that apoptosis of pulmonary artery smooth muscle cells in the HFD group was lower than that in the ND group (Fig. 4C). Moreover, the levels of PPAR-γ and apelin were lower in the lung tissue of the HFD group (Figs. 4D and 4E).

Discussion

In the current study, HFD suppressed pulmonary artery reverse remodeling and improvements of RVSP and RVH in hypoxia-induced PAH mice during reoxygenation. One of the possible mechanisms is the undiminished anti-apoptotic pulmonary smooth muscle cells and the reduced PPAR-γ levels in HFD. Although there have been several studies reporting that metabolic disorders are associated with the onset and progression of PAH in experimental models [18–20], the present study is the first to show that a HFD affects the improvement process of PAH after reoxygenation.

It has been reported by Khirfan et al. that plasma levels of HDL-C in PAH patients were lower than those in controls, and that low HDL-C was associated with PAH patient mortality [21]. In current study, mice fed with HFD exhibited higher HDL-C and RVSP. The discrepancy between the results of Khirfan et al.'s study and ours may be attributable to the differences between the development stage or the improvement stage of PAH.

Recently, Umar et al. reported that a western diet increased inflammation in the lungs, and led to the development of pulmonary hypertension in LDL receptor knockout mice [22]. In the present study, although inflammatory changes were not examined, TUNEL-positive cells in pulmonary artery smooth muscle cells and caspase-3 activity in the lung were decreased in the HFD group. Since the role of native or oxidized LDL for apoptosis or proliferation of smooth muscle cells is controversial [22–26], further study is needed. In this study, the HFD group presented higher RVSP and higher HDL-C levels than the ND group; however, previous studies have shown an association of higher plasma HDL-C levels with good prognosis in human PAH [27, 28]. However, the detailed underlying mechanisms responsible for the beneficial effects of HDL-C in pulmonary circulation are not fully explored [27], and a multicenter prospective cohort study by Cracowski et al. showed that HDL-C was not associated with survival in PAH [29]. Thus, the role of HDL-C level in PAH remains unclear.

Further, Umar et al. showed that a western diet affects left ventricular systolic pressure and RVSP [22]. However, in the present study, HFD did not affect the least left ventricular weight.

Chen et al. revealed that mitochondrial dysfunction represented by decreased ATP production in the lung induces hydrogen peroxide generation and is necessary for the apoptosis of smooth muscle cells in reverse remodeling during reoxygenation in hypoxia-induced PAH mice [11]. Our study showed that mitochondrial ATP production was higher in the HFD group than in the ND group. This suggests the possibility that the improvement in mitochondrial function by HFD may have acted suppressively on reverse remodeling.

Patients with PAH are reported to have lower levels of plasma-apelin, and the administration of apelin agonist improved the hemodynamics in PAH patients [10, 30, 31]. Apelin is regulated by PPAR-γ [10]; thus, it is suggested that the decreased PPAR-γ in reoxygenated-HFD mice causes the downregulation of apelin and delays pulmonary reverse remodeling.

Until now, the effectiveness of lipid lowering therapy for PAH in previous studies have been controversial, despite the association between metabolic disorder and PAH [32–34]. In fact, it is confirmed that an intervention for dyslipidemia is less effective than the use of pulmonary vasodilators. However, even though pulmonary vasodilators dramatically improve the prognosis of PAH patients [1, 4], there are cases in which these drugs cannot be used due to side effects. Therefore, it is necessary to establish a treatment other than vasodilator therapy. Further, we believe that with the improvement in the prognosis of PAH patients, complications of metabolic disorders increase. Our results suggest that treatment to metabolic disorder in addition to pulmonary vasodilator has a supportive effect for improving PAH.

Conclusion

In conclusion, we here for the first time reported that a high-fat diet delays reverse remodeling of pulmonary arteries of hypoxia-induced PAH mice. Metabolic disorder in PAH patients may require active treatment in order to improve pulmonary artery pressure as soon as possible.

Abbreviations

PAH: Pulmonary arterial hypertension

PPAR-γ: Peroxisome proliferator-activated receptor-γ

HFD: high-fat diet

RVSP: right ventricular systolic pressure

RVH: right ventricular hypertrophy

ND: normal diet

RV: right ventricle

LV: left ventricle

S: septum

α-SMA: α-smooth muscle actin

EM: Elastica-Masson

HDL-C: high-density lipoprotein cholesterol

LDL-C: low-density lipoprotein cholesterol

TG: triglyceride

ATP: adenosine triphosphate

TUNEL: terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study does not involve human participants, human material, or human data. All animal experiments were performed in compliance with the Fukushima Medical University Animal Research Committee. The investigations conformed to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th Edition, 2011), and our research protocol was approved by the Fukushima Medical University Animal Research Committee. The study on animals was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not Applicable.

Acknowledgements

Not Applicable.

Competing interests

Koichi Sugimoto and Tetsuro Yokokawa belong to a department supported by Janssen Pharmaceutical K.K. This company was not associated with the contents of this study. Tomofumi Misaka, Takashi Kaneshiro, Akiomi Yoshihisa, Kazuhiko Nakazato and Yasuchika Takeishi have no competing interests as defined by BMC.

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None.

Authors’ contributions

Conception: KS and YT. Design of the work: KS and YT. Acquisition of data: KS and TY. Analysis and interpretation of data: KS, TM, TK, and AY. Manuscript writing: KS, and YT. Review and editing: KN and YT. All authors have read and approved the final manuscript.

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Figures

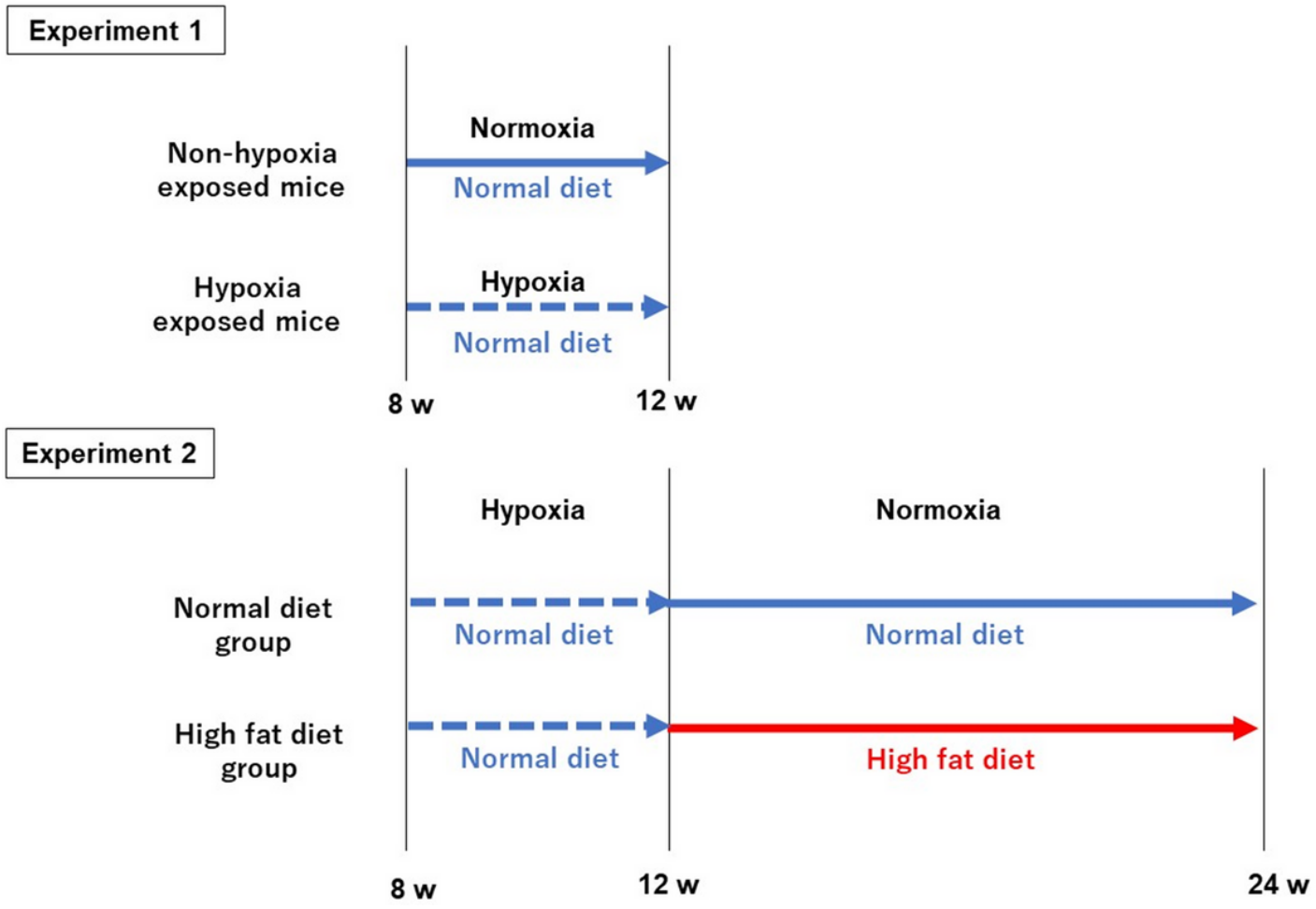

Figure 1

Experimental groups and protocols in mice. In Experiment 1, the mice were exposed to normoxia or hypoxia with a normal diet for 4 weeks. In Experiment 2, the mice were exposed to hypoxic condition for 4 weeks and divided into the normal diet and high fat diet group during reoxygenation (normoxia) for 12 weeks.

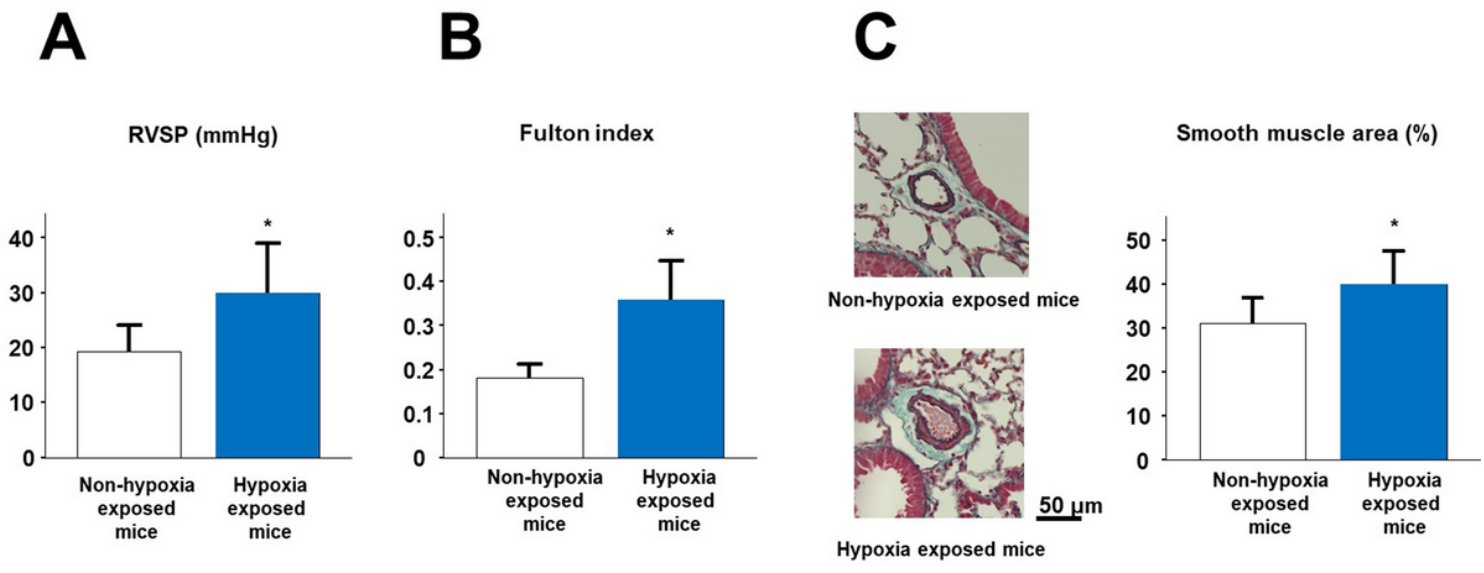

Figure 2

Effect of hypoxia on RVSP and RVH. Female C57BL/6 mice were exposed to normoxia or hypoxia for 4 weeks (Experiment 1). (A) RVSP measured by a micromanometer catheter, (B) Fulton index, (C) Representative microphotographs of the pulmonary arteries stained by EM staining. Smooth muscle area was calculated as described in the Methods. Results are expressed as mean ± S.D. of 8 to 10 animals. *P < 0.05 vs. normoxia group.

Supplementary Files

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