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# Adiponectin ameliorates hypoxia-induced pulmonary arterial remodeling

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ABSTRACT

We have demonstrated that adiponectin has anti-atherosclerotic properties. We also reported hypoadiponectinemia and nocturnal reduction in circulating adiponectin concentrations in patients of severe obstructive sleep apnea–hypopnea syndrome (OSAS). OSAS is often complicated with pulmonary hypertension. In this study, we investigated the effect of adiponectin on chronic hypoxia-induced pulmonary arterial remodeling in mice. Exposure of mice to 3-weeks sustained hypoxia (10% $O_2$) resulted in significant accumulation of adiponectin in pulmonary arteries. The percentage media wall thickness (%MT), representing pulmonary arterial remodeling, under hypoxic condition, was greater in adiponectin-knockout mice than wild-type mice. Overexpression of adiponectin significantly decreased hypoxia-induced pulmonary arterial wall thickening and right ventricular hypertrophy. These findings demonstrate for the first time that overexpression of adiponectin suppresses the development of hypoxic-induced pulmonary remodeling, and that adiponectin may combat a new strategy for pulmonary vascular changes that underlie pulmonary hypertension in OSAS.

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## Introduction

Adipose tissue is not only a passive reservoir for energy storage but also an important endocrine organ, secreting a variety of bioactive molecules (called adipocytokines), involved in energy metabolism, inflammatory response, and cardiovascular functions [1]. Aberrant production of adipocytokines plays a causative role in the development of metabolic syndrome, obesity-related disease clusters, including dyslipidemia, hypertension, and glucose intolerance, leading to atherosclerosis [2]. We identified adiponectin as an adipocytokine in the human adipose tissue cDNA library [3]. Hypoadiponectinemia is an independent risk factor for type 2 diabetes [4], hypertension [5] and cardiovascular disease [6], which are components of metabolic syndrome. Adiponectin exhibits direct anti-diabetic [7], anti-atherogenic [8], and anti- inflammatory properties [9]. Adiponectin-knockout mice develop severe diet-induced insulin resistance [7], increased neointimal thickening after balloon angioplasty [10], and excessive cardiac remodeling after pressure overload [11] compared to wild-type mice. Conversely, overexpression of adiponectin enhances insulin sensitivity [7], prevents arteriosclerosis [8], and inhibits cardiac hypertrophy [6]. Thus, adiponectin is an important key molecule in metabolic syndrome.

On the other hand, obstructive sleep apnea–hypopnea syndrome (OSAS) is closely linked with the metabolic syndrome [12] and is potentially associated with insulin resistance and hypertension, leading to cardiovascular disease [13]. Moreover, repetitive episodes of apnea during sleep result in arterial hypoxemia, hypercapnia, increased sympathetic tone, surge in pulmonary blood pressure due to pulmonary vasoconstriction, and pulmonary arterial remodeling, which ultimately results in right ventricular (RV) hypertrophy [14]. Our recent report showed daytime hypoadiponectinemia and nocturnal falls in circulating adiponectin concentrations in severe OSAS subjects, partially due to hypoxic stress [15]. Dysregulation of adiponectin in OSAS may play a role in OSAS-related diseases, such as hypertension, insulin resistance, glucose intolerance, and pulmonary hypertension. Adiponectin has been reported to provide several protective effects against OSAS-related diseases [7–9], but its effect on hypoxia-induced pulmonary arterial remodeling remains to be elucidated. In this study, we investigated the effect of adiponectin on hypoxia-induced pulmonary arterial remodeling in mice.

## Materials and methods

*Animals and exposure to hypoxia.* Male wild-type (WT) C57BL/6J and adiponectin-knockout (adipo-KO) mice (each group; $n = 6$) were obtained from Clea Japan (Tokyo, Japan) and kept under a 12–12 h dark–light cycle (lights on 8:00 A.M. to 8:00 P.M.) and constant temperature (22 °C) with free access to food (Oriental Yeast, Osaka, Japan) and water. At the age of 12 weeks, male mice were housed in cages exposed to room air (ambient atmosphere) or in hypoxia chambers (Teijin Pharma, Osaka, approximately 10% $O_2$) for 3 weeks. This $O_2$ concentration is often used in hypoxia stress studies [15,16]. The chamber was opened every 7 days for

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feeding and changing animal cages. After closing the chamber, the mixed gas was flushed to re-instate the hypoxic environment as quickly as possible.

After 3-weeks exposure to hypoxia, the mice were sacrificed under pentobarbital sodium anesthesia (50 mg/kg body weight), and then various tissues were harvested for analysis, and blood samples were collected for measurement of adiponectin using enzyme-linked immunosorbent assay (ELISA), as described previously [17]. The experimental protocol was approved by the Institutional Laboratory Animal Care and Use Committee of Osaka University.

*Preparation and administration of adenovirus.* Adenovirus producing the full-length mouse adiponectin was prepared by using the Adenovirus Expression Vector kit (Takara, Kyoto, Japan). At the age of 12 weeks, $1.65 \times 10^7$ plaque-forming units of adenovirus-adiponectin (Ad-APN) or adenovirus-β-galactosidase (Ad-βgal) was injected into the tail vein of WT mice at 5 days prior to exposure to hypoxia. After 5 days of injections, mice were housed in cages exposed to room air (ambient atmosphere) or in the same hypoxia chambers described above for 2 weeks. On the 20th day after the virus injection (14th day after injury), the mice were sacrificed, and then analyzed as described above.

*Immunohistochemistry.* Lung tissues were sampled after injection of 4% paraformaldehyde through the trachea to expand the alveoli. Lungs were fixed with 4% paraformaldehyde, embedded in paraffin, and subsequently cut into 4 μm-thick sections. Sections were analyzed immunohistochemically by using rabbit polyclonal anti-mouse adiponectin antibody (Otsuka Pharmaceutical) as described previously [18], and stained with hematoxylin and eosin (H&E). Smooth muscle cells were identified by immunostaining for α-smooth muscle actin (αSMA) (Dako, Glostrup, Denmark) as the primary antibody. After incubation with biotin-conjugated secondary antibody, as to adiponectin antibody, the specimens were processed by using the avidin–biotin–peroxidase complex kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with a Liquid DAB Substrate Kit (Zymed Laboratories Inc., San Francisco, CA). More than 20 consecutive sections in each mouse were examined, and the percentage of media wall thickness (%MT) and external diameter were calculated. The number of cells was determined from light microscopic images (Provis AX 80 equipped with an HDTV system and a color-chilled 3 charged coupled device camera; Olympus) using an image analysis system (Win ROOF version5.5, Mitani, Japan).

*Quantification of pulmonary vessel damage.* The morphological changes in small pulmonary arteries (external diameter: 10–60 μm) were estimated by %MT, which was calculated by [(media thickness × 2/external diameter) × 100], as described previously [16,19,20]. The width of the media and external diameter were measured using image analysis software (Win ROOF version5.5, Mitani). %MT was calculated for at least 40 small pulmonary arteries in each mouse, and the shortest external diameter was measured to allow for vessels not being perfectly symmetrical circles.

*Statistical analysis.* All values were expressed as means ± SEM. Comparisons among groups were made by one- or two-way analysis of variance (ANOVA) with Fisher's protected least significant difference test for multiple-group analysis or unpaired Student's *t*-test for experiments of only two groups. Probability values of $p < 0.05$ were considered to indicate statistical significance. All statistical analyses were performed with StatView-J 5.0 (Statistical Analysis System Inc., Cary, NC).

## Results

### *Accumulation of adiponectin in pulmonary vessels under control and hypoxic conditions*

Immunohistochemical analysis showed abundant immunostaining for adiponectin in the endothelium of large (Fig. 1 top: external diameter: 50–80 μm) and small pulmonary vessels (Fig. 1 bottom: external diameter: 20–50 μm) of wild-type (WT) mice exposed to hypoxia (Fig. 1 middle) for 3 weeks but not in that of control vessels (Fig. 1 left). There was no accumulation of adiponectin in adipo-KO mice (Fig. 1 right). Real-time PCR showed no detectable level of adiponectin mRNA in the vessels of WT mice (data not shown). These findings indicate accumulation of adiponectin in pulmonary vessels of hypoxia-exposed mice.

### *Effect of hypoxic exposure on pulmonary vessels and heart in adiponectin-knockout mice*

First, we investigated the effect of exposure to sustained hypoxia on pulmonary arterial remodeling in WT and adipo-KO mice. In WT mice, exposure to hypoxia for 3 weeks resulted in significant suppression of serum adiponectin concentrations compared to the control condition (hypoxia, 10.1 ± 0.7; control, 13.4 ± 0.8 μg/mL, $p < 0.05$), as reported previously [15], while

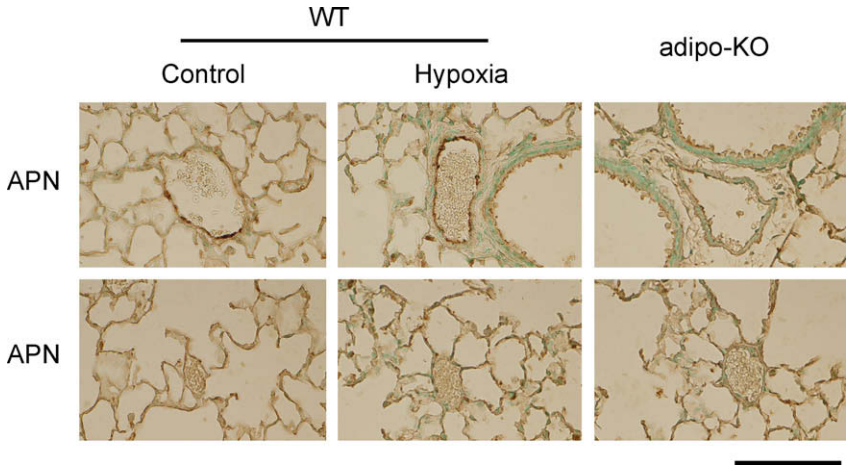


**Fig. 1.** Representative results of immunohistochemical analyses for adiponectin in pulmonary vessels of wild-type (WT) mice under control and hypoxic conditions and adiponectin-knockout (adipo-KO) mice (negative control, right). (Top) larger vessel (external diameter, 50–80 μm), (bottom) smaller vessel (external diameter, 20–50 μm). Adiponectin (APN) staining. Control mice showed limited pulmonary endothelial staining for adiponectin (left), whereas immunostaining for adiponectin was augmented after hypoxic stress (middle). Magnification; ×400, scale bar; 100 μm. Similar results were observed in other series of experiments.

serum adiponectin concentrations were undetectable under control and hypoxic conditions in adipo-KO mice (data not shown). Analysis of small pulmonary vessels showed no significant differences in pulmonary artery diameter under both control and hypoxic conditions between WT and adipo-KO mice (each median value, WT(control); 28.0, WT(hypoxia); 28.5, adipo-KO(control); 28.9, adipo-KO(hypoxia); 29.7 μm, Fig. 2A). We next investigated the morphology of small pulmonary vessels (external diameter: 10–60 μm) in WT and adipo-KO mice under control and chronic hypoxic conditions. Examination of H&E-stained and αSMA-stained sections of peripheral small pulmonary arteries of WT and adipo-KO mice under control and hypoxic conditions, revealed that chronic hypoxia resulted in pulmonary arterial wall thickening in both WT and adipo-KO mouse (Fig. 2B). Quantitative analysis of these sections showed that %MT, as a marker of morphological changes in small pulmonary arteries, was significantly greater under hypoxic conditions than control conditions in WT mice (Fig. 2C left, control; 15.0 ± 0.6%, hypoxia; 21.8 ± 0.4%, $p < 0.0001$). Chronic hypoxia also induced pulmonary arterial wall thickening in adipo-KO mice (control; 17.9 ± 1.3%, hypoxia; 23.4 ± 0.6%, $p < 0.001$, Fig. 2C left). Interestingly, under hypoxic conditions, %MT was greater in adipo-KO mice than in WT mice ($p < 0.05$, Fig. 2C left). There was a trend for increase in %MT in adipo-KO mice, compared to WT mice, under control conditions, although the change was not significant ($p < 0.05$, Fig. 2C left). The external diameters of small pulmonary vessels were not different between the groups under either control or hypoxia (WT(control); 30.3 ± 1.8, WT(hypoxia); 28.7 ± 0.6,

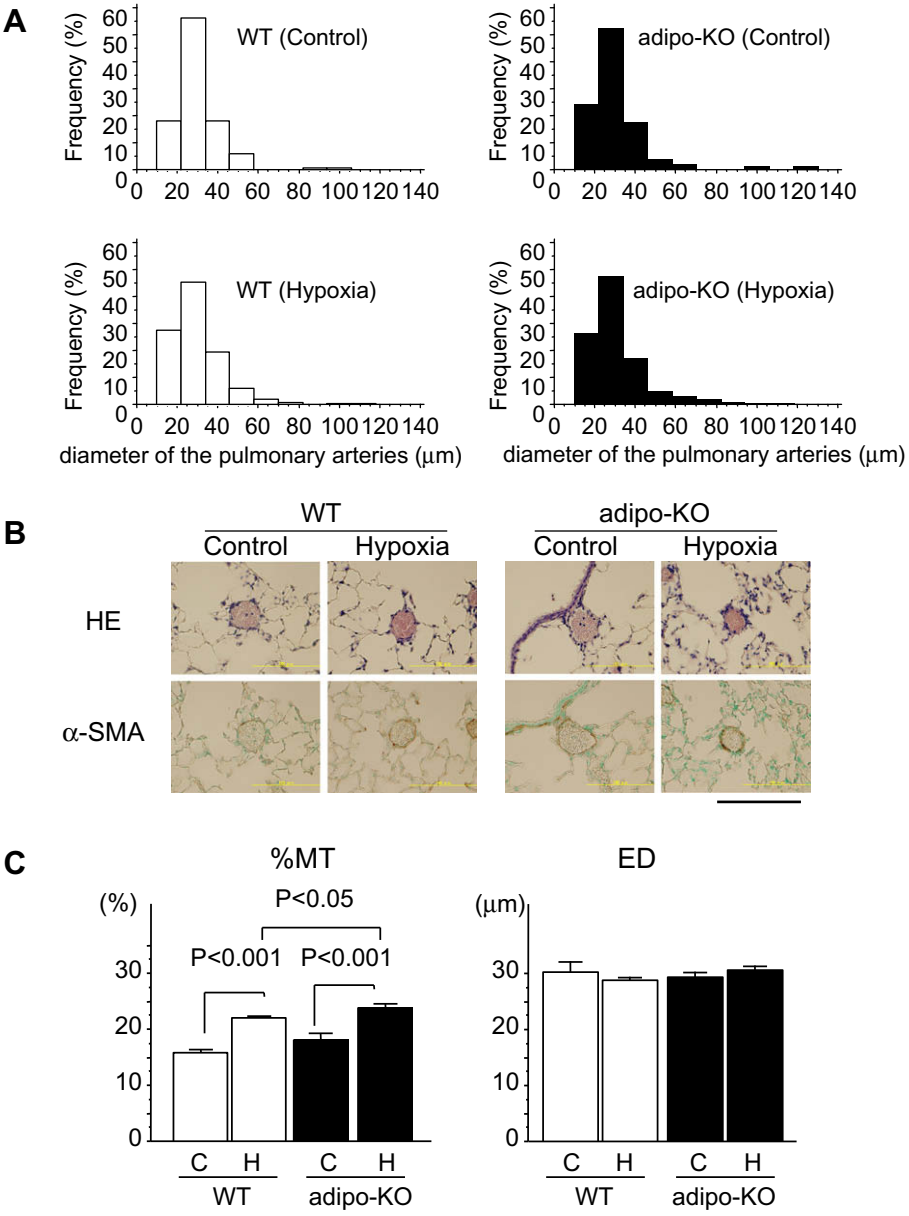


**Fig. 2.** Effects of hypoxic exposure for 3 weeks on pulmonary arterial remodeling in WT and adipo-KO mice. (A) Distribution of pulmonary artery diameter in wild-type (WT) and adiponectin-knockout (adipo-KO) mice under control and hypoxic conditions for 3 weeks. Control (C); room air condition, Hypoxia (H); 10% $O_2$ concentrations. Magnification; ×400, scale bar; 100 μm. (B) Upper panels: hematoxylin and eosin (H&E) staining. Lower panels: α-smooth muscle actin (α-SMA) staining. Magnification; ×400, scale bar; 100 μm. (C) Left: calculated %MT, right: external diameter, of small pulmonary arteries in WT and adipo-KO mice under control (C) and chronic hypoxic (H) conditions for 3 weeks. Number of mice in each group = 6. (C) right; external diameter of small pulmonary arteries was 10–60 μm. About 200 vessels were analyzed in each group. %MT (media × 2/ED × 100), ED, external diameter. Similar results were observed in other series of experiments. Data are mean ± SEM.

adipo-KO(control); 29.4 ± 0.6, adipo-KO(hypoxia); 30.6 ± 0.9 μm, Fig. 2C right). To assess the effect of chronic hypoxia on the RV, we determined the ratios of the RV free wall and the left ventricular free wall plus septum (LV + S) in WT and adipo-KO mice, under control and hypoxic conditions, as described previously [19]. The RV/LV + S (mg/mg) ratio of hypoxic WT mice was greater than that of normoxic controls (WT control, 0.267 ± 0.012; WT hypoxia, 0.368 ± 0.028, $p < 0.05$). The same pattern was noted in adipo-KO mice (KO control, 0.272 ± 0.015; KO hypoxia, 0.398 ± 0.042, $p < 0.05$), suggesting RV hypertrophy is induced by pulmonary hypertension in both strains of mice. There was no significant difference in RV/LV + S ratio between WT and adipo-KO mice under chronic hypoxic conditions.

*Adenovirus-mediated overexpression of adiponectin attenuates hypoxia-induced pulmonary artery thickening*

WT mice were infected with adenovirus (Ad)-βgal or Ad-adiponectin (APN) prior to hypoxic exposure. Ad-APN infection resulted in a 4- to 5-fold increase in serum levels of adiponectin on the 14th day after adenoviral injection in WT mice compared with those in Ad-βgal-infected WT mice (Ad-βgal(control); 16.1 ± 0.7 versus Ad-APN(control); 69.1 ± 16.1, Ad-βgal(hypoxia); 12.8 ± 0.3 versus Ad-APN(hypoxia); 69.8 ± 11.3 μg/mL, $p < 0.01$, respectively). Analysis of pulmonary vessels showed that there were no significant differences in the diameter of pulmonary arteries under control and hypoxic conditions between Ad-βgal and Ad-APN injected mice

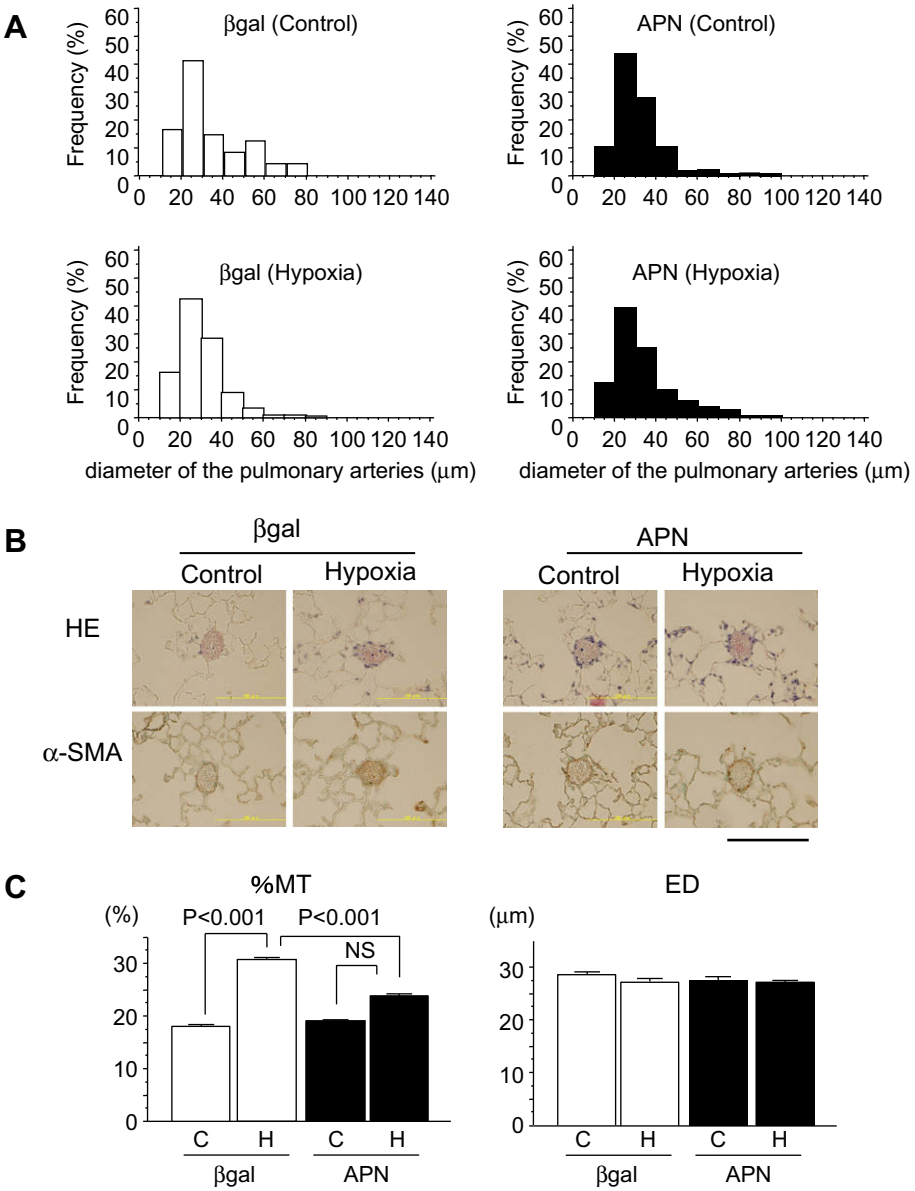


**Fig. 3.** (A) Distribution of diameter of pulmonary arteries in adenovirus-β-galactosidase (Ad-βgal)- and adenovirus-adiponectin (Ad-APN)-infected mice under control and hypoxic conditions. See Fig. 2A for more details. (B) Representative results of immunohistochemical analyses for adiponectin in pulmonary vessels of Ad-βgal- and Ad-APN-infected mice under control and hypoxic conditions (10% $O_2$ concentrations). Top panels: hematoxylin and eosin (H&E) staining. Lower panels: α-smooth muscle actin (α-SMA) staining. Hypoxic stress resulted in pulmonary vascular remodeling in both Ad-βgal- and Ad-APN-infected mice. (Magnification; ×400). Similar results were obtained in other series of experiments. (C) The calculated %MT (left) and external diameter (ED) (right), of small pulmonary arteries (external diameter of small pulmonary arteries was 10–60 μm) in Ad-βgal- and Ad-APN-infected mice under control and chronic hypoxic conditions for 2 weeks. Number of mice in each group = 6. The calculated %MT and ED of 200 vessels were analyzed in each group. Data are means ± SEM. Similar results were observed in other series of experiments. NS, not significant.

(Fig. 3A), each median value of the diameter was, 28.0 (Ad-βgal(control)), 26.9 (Ad-βgal(hypoxia)), 26.4 (Ad-APN(control)), and 26.7 μm (Ad-APN(hypoxia)). Fig. 3B shows representative H&E- and αSMA-stained sections of the peripheral pulmonary arteries in Ad-βgal- and Ad-APN-infected mice under control and hypoxic conditions. Quantitative analysis of these sections revealed that %MT under hypoxic conditions were significantly greater than under control conditions in Ad-βgal-injected mice (Ad-βgal(control); 18.1 ± 0.3%, Ad-βgal (hypoxia); 30.1 ± 0.5%, $p < 0.001$, Fig. 3C left). There was no significant difference in %MT of Ad-APN-injected mice between control and hypoxic conditions (Ad-APN(control); 18.8 ± 0.5%, Ad-APN(hypoxia); 24.5 ± 0.3%, Fig. 3C left). Overexpression of adiponectin in Ad-APN-injected mice significantly suppressed pulmonary arterial wall thickening, compared to Ad-βgal-injected mice under hypoxic stress (Ad-βgal(hypoxia) versus Ad-APN(hypoxia) $p < 0.001$, Fig. 3C left). The external diameters of small pulmonary vessels were not different between the groups (Ad-βgal(control); 29.1 ± 0.6, Ad-βgal(hypoxia); 28.6 ± 0.6, Ad-APN(control); 27.8±0.8, Ad-APN(hypoxia; 27.9 ± 0.5μm, Figure 3C right). The RV/LV + S (mg/mg) ratio in hypoxic Ad-βgal-infected mice was greater than in normoxic controls (Ad-βgal(control); 0.256 ± 0.015, Ad-βgal(hypoxia); 0.389 ± 0.016, $p < 0.01$). Furthermore, overexpression of adiponectin attenuated significantly the hypoxia-induced RV hypertrophy (Ad-βgal(hypoxia); 0.389 ± 0.016, Ad-APN(hypoxia); 0.310 ± 0.021, $p < 0.05$). These results suggest that adiponectin attenuates hypoxia-induced RV hypertrophy.

## Discussion

The major findings of the present study are; (1) %MT, which represents pulmonary arterial remodeling, was greater in adipo-KO mice than in WT mice under chronic hypoxia; (2) adiponectin accumulated in the endothelial linings of hypoxic pulmonary arteries; (3) overexpression of adiponectin attenuated chronic hypoxia-induced pulmonary arterial wall thickening and RV hypertrophy.

Chronic hypoxia leads to pulmonary vascular remodeling and RV hypertrophy [19,21,22]. Accumulation of adiponectin in hypoxic pulmonary arteries might protect against hypoxia-induced pulmonary vascular remodeling (Fig. 1), since our previous immunohistochemical studies showed that adiponectin adheres to the injured arterial wall [8]. We also reported that physiological concentrations of human recombinant adiponectin suppressed the expression of endothelial adhesion molecules, vascular smooth muscle cell proliferation, macrophage-to-foam cell transformation, and tumor necrosis factor-α production by macrophages *in vitro* [10,23–25], and recently reported downregulation of adiponectin in adipose tissues of mice under chronic hypoxia [15]. The present results showed that adiponectin ameliorated hypoxia-induced pulmonary vascular remodeling and RV hypertrophy. Hypoxia-induced pulmonary arterial remodeling was consisted with smooth muscle proliferation as demonstrated in this study. In hypoxic condition, adipo-KO mice showed greater %MT, and overexpression of adiponectin attenuated hypoxia-induced pulmonary arterial remodeling. Our histological findings did not clarify what component was responsible for the hypoxia-induced pulmonary vascular thickening, because the vessels analyzed were too small to allow identification of vessel wall component of the remodeling vessels, but adiponectin might suppress pulmonary vascular smooth muscle cell proliferation, as described previously *in vitro* [10].

Chronic hypoxia causes pulmonary hypertension due to pulmonary arterial remodeling, which often results in RV hypertrophy [21]. The present study demonstrated no significant difference between RV/LV+S ratio in WT and adipo-KO mice under chronic hypoxic conditions, because exposure to hypoxia resulted in significant suppression of serum adiponectin concentrations compared to the control condition, as reported previously [17]. It is possible that overexpression of adiponectin might ameliorate hypoxia-induced RV hypertrophy, although further studies are necessary to elucidate the precise mechanism.

To clarify the mechanism, we analyzed the expression levels of several genes related to vascular remodeling and cardiac hypertrophy. Under hypoxic condition, the mRNA levels of endothelial nitric oxide synthase, inducible nitric oxide synthase, platelet-derived growth factor-β and collagen type I/III were significantly elevated, compared to control, in both Ad-βgal- and Ad-APN-infected mice, as reported previously in smooth muscle α-actin thickening in the artery [10,25,26]. However, there was no significant difference in the expression levels of these genes between Ad-βgal- and Ad-APN-infected mice under hypoxic condition. In Ad-βgal- and Ad-APN-infected mice, hypoxia also did not alter the mRNA levels of α-SMA, transforming growth factor beta 1, hypoxia-inducible factor-1α and nuclear factor of activated T cells isoform c3, which are known to be affected by hypoxic stress [25,27,28] (data not shown). The lack of difference in the present study might be due to studying the whole lung tissue including alveoli and other tissues. Further *in vitro* studies should examine changes in these genes in pulmonary artery smooth muscle cell lines and pulmonary artery endothelial cell lines.

In conclusion, we demonstrated for the first time accumulation of adiponectin in pulmonary vessels exposed to hypoxia, and that adiponectin ameliorates hypoxia-induced pulmonary arterial remodeling. Our results suggest that increasing serum adiponectin concentrations might be therapeutically beneficial in OSAS-related disorders.

## Disclosures statement

The authors declare no conflict of interest.

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