Absence of the Adenosine A_{2A} Receptor Confers Pulmonary Arterial Hypertension and Increased Pulmonary Vascular Remodeling in Mice

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Key Words

Adenosine A_{2A} receptor • Collagen hyperplasia • Pulmonary arterial hypertension • Pulmonary vascular remodeling • Smooth muscle hypertrophy

Abstract

Background: Pulmonary arterial hypertension (PAH) is characterized by sustained elevation of pulmonary vascular resistance resulting from endothelial and smooth muscle cell dysfunction and collagen deposition in pulmonary vascular walls. In this study, we investigated the role of the adenosine A_{2A} receptor (A_{2A}R) in the development of PAH by determining the effect of genetic inactivation of A_{2A}Rs on pulmonary vascular remodeling in mice. **Methods and Results:** We characterized hemodynamic, histological and ultrastructural changes in pulmonary vascular remodeling in A_{2A}R knockout (KO) mice compared with their wild-type (WT) littermates after exposure to normoxia and hypoxic conditions. After exposure to normoxia, compared to WT mice, A_{2A}R KO mice displayed: (1) increased right ventricular systolic pressures and an elevated ratio of the right ventricle over left ventricle plus septum (Fulton index), (2) increased wall area and thickness as well as enhanced smooth muscle actin immunoreactivity in pulmonary resistance vessels, (3) in-

creased proliferating cell nuclear antigen-positive cells in pulmonary resistance vessels and (4) increased smooth muscle cells hypertrophy and collagen deposition in the adventitia of pulmonary arteriole walls as revealed by electron microscope. By contrast, histological analysis revealed no features of hypertensive nephropathy in A_{2A}R KO mice and there was no significant difference in systemic blood pressure, and left ventricular masses among the 3 genotypes. Furthermore, following chronic exposure to hypoxia, A_{2A}R KO mice exhibited exacerbated elevation in right ventricular systolic pressure, hypertrophy of pulmonary resistance vessels and increased cell proliferation in pulmonary resistance vessels, compared to WT littermates. Thus, genetic inactivation of A_{2A}Rs selectively produced PAH and associated increased smooth muscle proliferation and collagen deposition. **Conclusions:** Extracellular adenosine acting at A_{2A}Rs represents an important regulatory mechanism to control the development of PAH and pulmonary vascular remodeling.

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Introduction

Pulmonary arterial hypertension (PAH) develops as a primary disease with an unclear underlying cause or occurs as a complication of various pathological conditions (such as chronic exposure to hypoxia at high altitude) [1]. Pulmonary vascular remodeling with abnormal sclerosis/stenosis of distal pulmonary arteries produces progressive elevation of pulmonary vascular resistance and pulmonary arterial pressure, followed by impaired right ventricular output. The morphological changes consist of common hypertrophy of the tunica media, proliferative lesions such as intimal thickening or plexiform lesions and in situ thrombosis [2]. The expansion of the tunica media in pulmonary arteries in PAH occurs by a combination of hypertrophy and hyperplasia. Hypertrophy of medial smooth muscle cells (SMCs) is a characteristic pathological feature of PAH that involves muscularized arteries and precapillary vessels. In addition, the collagen deposition in the pulmonary vascular wall is another important characteristic of pulmonary vascular remodeling. The increase in collagen expression and disordered proteolysis of extracellular matrix likely contributes to pathological pulmonary arterial structural remodeling and loss of vasoreactivity [3]. Under certain conditions, adventitial fibroblasts are activated and proliferate and upregulate the expression of collagen and extracellular matrix proteins. Thus, the development of PAH involves a complex interplay of multiple genetic, environmental and hormonal abnormalities, leading to abnormal pulmonary vascular remodeling involving endothelial cells, smooth muscle and fibroblasts. However, the physiological regulator of PAH development remains to be determined.

Adenosine is an important intermediate of purine and energy (ATP) metabolisms, and is ubiquitously distributed throughout the body. Endogenous adenosine levels vary from tissue to tissue from 10 to 100 nM, with adenosine levels being higher in the pulmonary than the systemic circulation [4]. For example, SMCs synthesize a substantial amount of adenosine [5]. Importantly, extracellular adenosine levels increased markedly in response to hypoxia [6]. However, patients with PAH have lower plasma adenosine levels in the pulmonary circulation compared to control subjects, indicating a possible deficiency in adenosine signaling in PAH [4]. Adenosine acting through multiple G protein-coupled receptors, namely A_1 , A_{2A} , A_{2B} and A_3 , exert important regulatory effects on various physiological and pathophysiological processes, including suppression of immune response and inflammation, tissue protection, vasodilatation and an-

giogenesis, neuromodulation and sleep promotion [7]. Immunohistochemical analysis of lung parenchyma demonstrated A_{2A} receptor ($A_{2A}R$) expression in bronchiolar and alveolar epithelial cells, SMCs localized in bronchiolar and vessel walls, and endothelial cells in pulmonary arterials [8]. On the other hand, the A_{2B} receptor was expressed mainly in mast cells and macrophages and the A_1 receptor was expressed only in a few alveolar macrophages [8].

Several lines of experimental evidence suggest that adenosine may be a potential endogenous regulator of PAH development by regulating SMC growth and collagen synthesis and by maintaining vascular homeostasis in systemic circulation systems. First, adenosine acting at $A_{2A}R$ is a powerful vasodilator for systemic arterial pressure with possible similar effects on pulmonary arterials [9]. Importantly, this effect does not require a functional endothelium [10], since adenosine can induce significant pulmonary vasodilation under conditions of severe nitric oxide deficiency and endothelial dysfunction, that is, conditions seen in severe PAH [11]. Second, adenosine acting at $A_{2A}R$ is also a powerful stimulator of angiogenesis and is responsible for up to 50–70% of hypoxia-induced angiogenesis under certain pathological conditions [12]. Third, exogenous adenosine inhibited SMC growth induced by fetal calf serum [13]. Fourth, adenosine may contribute to the abnormal synthesis and deposition of collagen observed in vascular-occlusive disorders associated with hypertension, atherosclerosis and restenosis [2, 14]. For instance, adenosine acting through the cAMP pathway inhibits collagen synthesis and hypertrophy of vascular SMCs and cardiac fibroblasts [15]. Based on these findings, we hypothesized that adenosine acting at $A_{2A}R$ regulates the pulmonary vascular remodeling (particularly SMCs and fibroblasts) and contributes to the development of PAH.

In this study, we critically evaluated the contribution of $A_{2A}R$ to the development of PAH using $A_{2A}R$ knockout (KO) mice. Development of the $A_{2A}R$ KO mice, particularly the $A_{2A}R$ KO mice in the congenic C57BL/6 background to reduce potential genetic background effect [16, 17], circumvents intrinsic limitations of partial specificity of $A_{2A}R$ pharmacologic agents and provides a unique opportunity to investigate $A_{2A}R$ in pulmonary vascular remodeling [18]. Using the $A_{2A}R$ KO mice, we provided the first experiment evidence at hemodynamic, histological and ultrastructural levels that genetic deletion of the $A_{2A}R$ confers a selective hypertensive and hypertrophic phenotypes of pulmonary circulation in $A_{2A}R$ KO mice.

Materials and Methods

Generation and Genotyping of A_{2A}R KO Mice

The A_{2A}R KO mice were developed by Chen et al. [16] and described previously. Congenic A_{2A}R KO mice in a C57BL/6 background were generated by backcrossing A_{2A}R KO mice in mixed 129-Steel × C57BL/6 background to C57BL/6 mice for >10 generations [18], and used for this study. Heterozygous crossbreeding was used to generate homozygous A_{2A}R KO and wild-type (WT) mice, all from the same breeding pairs. The A_{2A}R KO and WT mice at the age of 14–16 weeks were used for this study. The genotypes of the mice were determined by PCR analysis using the 3 primer sets targeting to the neocassettes and the adjacent A2AR genes as described previously [18, 19]. A total of 80 arterioles from 20 sections/20 mice were analyzed by an investigator who was blinded to the genotypes.

Experimental Model of Pulmonary Hypertension after Chronic Exposure to Hypoxia

A_{2A}R KO and WT mice were exposed to chronic hypoxia (9–11% O₂) in a sealed but ventilated chamber as described previously [20]. The hypoxic environment was established by flushing the chamber with a mixture of room air and nitrogen, and the gas was recirculated. The chamber environment was monitored using an oxygen analyzer. Carbon dioxide was removed by soda lime granules. Normoxic mice, both A_{2A}R KO and WT, were kept in a similar chamber flushed with normoxic gas (21% oxygen) in the same room. After 2 weeks of exposure, mice were measured for the right ventricular systolic pressure (RVSP), and lungs were processed for morphological and histological examination.

Hemodynamic Measurements of RVSP

Mice were anesthetized with intraperitoneal sodium pentobarbital (85 mg/kg). After dissection to expose the right jugular vein, a 1.4F Millar Mikro-Tip pressure catheter (Millar Instruments) was inserted into the vein and advanced to the right ventricle (RV). The catheter was connected to a transducer unit interfaced with a signal amplifier and recorder (Powerlab Systems; AD Instruments). Following a 5-min stabilization period, RVSP and heart rate were recorded. We also monitored systemic blood pressure (SBP) by catheterization via the carotid artery. The investigator who was blinded to the genotypes performed all measurements.

Assessment of RV Hypertrophy Morphometrical Analysis of Pulmonary and Renal Arteries

The RV was dissected from the left ventricle plus septum and each component was weighed separately. The Fulton index (RV/left ventricle plus septum) was calculated as previously described [18]. All measurements were also made by an investigator who was blinded to the genotype.

Pulmonary and renal arteries were perfused with 4% paraformaldehyde via a cannula at a perfusion pressure of 20 cm H₂O. The left lungs were fixed in 4% paraformaldehyde, and then dehydrated in increasing grade of ethanol (30, 50, 70, 80, 95 and 100%). After delipidation with xylene, the lungs were embedded in paraffin and cut into 3–4 transverse sections. Lung sections (4 μm) were stained with hematoxylin and eosin (HE) and with the Elastica-van Gomori method. Pulmonary vascular remodeling was assessed by measuring the percentage of wall thickness

and wall areas of the arteries [21]. Five random fields (with 1–2 arterioles) from one section of each mouse were chosen and arterioles with diameters between 25 and 100 μm and being associated terminal bronchioli or respiratory bronchioli or alveolar ducts or alveolar walls were analyzed for their wall thickness, wall areas, external diameter and external areas. The wall thickness and wall areas of a total of 131 arteries/arterioles from 26 sections of 26 mice were analyzed using a computer imaging analysis. Wall thickness 1 was measured at one point of the vessel wall and wall thickness 2 at the diametrically opposite point, and the diameter was the shortest one across the center of the vessels. The percentage of wall thickness (% wall thickness) was calculated as [(wall thickness 1) + (wall thickness 2)] × 100/external diameter. External wall areas were measured as wall areas 1 and internal wall areas were measured as wall areas 2. The percentage of wall areas (% wall areas) was calculated as [(wall areas 1) – (wall areas 2)] × 100/wall areas 1. All morphometric analyses were performed by the investigator who was blinded to the genotype.

The sections (4 μm) were stained with HE and Elastica-van Gomori's staining for evidence of any structural abnormalities, such as fibrosis and thickening of the alveolar septa, or for any evidence of edema that may have occurred secondary to left ventricular dysfunction.

Detection of Expression of A_{2A}R, α-Smooth Muscle Actin and Proliferating Cell Nuclear Antigen in Pulmonary and Renal Arteries by Immunohistochemistry

A_{2A}R expression in pulmonary arteries of WT and A_{2A}R KO mice was detected by immunofluorescence immunohistochemistry using A_{2A}R antibody conjugated with FITC secondary antibody. Briefly, the frozen sections of lung tissues were acetone fixed for 10 min, followed by incubation on individual slides for 30 min at 37°C with rabbit anti-mouse A_{2A}R antibody (Santa Cruz; 1:300). After washing 3 times with PBS and incubating with FITC-conjugated goat anti-rabbit secondary antibody for 30 min at 37°C, the sections were washed in a similar manner and A_{2A}R-positive cells were viewed under fluorescence microscopy at 200× magnification. Total cells/nuclei in the same section were stained with 4'-6-diamidino-2-phenylindole (DAPI).

The sections were immunostained with monoclonal antibody to α-smooth muscle actin (α-SMA), a marker for SMCs (Chemicon; 1:500 dilutions) to demonstrate the expression of SMCs. Quantitative immunohistochemical assessments were performed as previously reported [22]. The integrated optical density, which relates to immunohistochemical staining intensity and area, was calculated in the vessel wall of pulmonary arteries and afferent glomerular arteries.

The lung sections were also immunostained with antibodies against proliferating cell nuclear antigen (PCNA; Boster Biological Technology; 1:100 dilutions) to determine cell proliferation in pulmonary arterial walls (arterioles 25–100 μm in diameter). PCNA, a 37-kDa molecular weight protein, was originally identified as an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle [23]. The cells positive for PCNA staining were brown in nuclei. We counted the numbers of positive cells and all the cells in the arterial walls. The percentage of positive cell number was calculated as positive cells/all cells.

Ultrastructural Examination of Pulmonary Arteries

Lung tissues of WT and A_{2A}R KO mice (n = 3 per group) were collected proximal to hilus pulmonis and immersed immediately in 3% glutaral solution in 0.2 M phosphate buffer. The tissues were processed further for transmission electron microscopy examination by postfixation with 2% osmium tetroxide followed by dehydration in acetone series and embedding. Grids were stained with 2% uranyl acetate followed by lead citrate. Ultrastructural images of at least 1 arteriole per sample were recorded and photographed on a Hitachi H-7500 electron microscope (Hitachi). The ultrastructural features and appearance of pulmonary vascular walls for each group were evaluated by transmission electron microscopy.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. The comparison among 3 genotypes was analyzed by one-way ANOVA, followed by post hoc comparison with LSD test (equal variances assumed) or Dunnett's test (equal variances not assumed). Two-way ANOVA was used to analyze the effect of genotypes (WT vs. KO) and oxygen condition (normoxia vs. hypoxia) and their interaction. A value of $p < 0.05$ is accepted as statistically significant.

Results

Expression of A_{2A}R in Pulmonary Arteries

To better characterize the role of A_{2A}R in the development of pulmonary hypertension in mice, we first determined the expression pattern of A_{2A}R in small and large pulmonary arterials of WT mice by fluorescence immunohistochemistry, and confirmed the specificity of the A_{2A}R immunoreactivity using A_{2A}R KO mice. A_{2A}R-positive cells were detected in both small pulmonary arteries (fig. 1a) and large pulmonary arteries (fig. 1b) of WT mice lung sections. Notably, most of these cells were detected in the tunica intima and tunica adventitia of pulmonary artery, indicating that their cell type may be endothelial cells and fibroblasts. Importantly, in both small pulmonary arteries (fig. 1c) and large pulmonary arteries (fig. 1d) of A_{2A}R KO mice, there was no A_{2A}R-positive cell in lung sections, validating the specificity of A_{2A}R immunoreactivity by fluorescence immunohistochemistry.

A_{2A}R KO Mice Exhibited Elevation in RVSP and Hypertrophy of RV

At the age of 14–16 weeks, the mean RVSP in WT mice was 28.20 ± 1.86 mm Hg, consistent with the measurement in the previous studies using C57BL/6 mice. Interestingly, the RVSP in A_{2A}R KO mice was 40.86 ± 2.80 mm Hg, increasing by 44.89% compared to the WT littermates ($p < 0.01$; fig. 2a). Figure 2b shows the trace recording of RVSP in 3 genotypes, with the RVSP in A_{2A}R KO markedly higher than that of A_{2A}R+/- and A_{2A}R KO

mice. In contrast to enhanced RVSP, there was no significant difference in mean SBP among 3 genotypes (WT mice 80.09 ± 11.79 mm Hg; A_{2A}R KO mice 94.46 ± 13.73 mm Hg). The difference in heart rate was not significant among 3 genotypes (WT mice 330.80 ± 61.16 bpm; A_{2A}R+/- mice 276.25 ± 35.62 bpm; A_{2A}R KO mice 326.71 ± 21.50 bpm). Thus, genetic deletion of A_{2A}R selectively produces PAH without affecting systemic circulation or heart rate.

To seek additional evidence in support of PAH phenotype, we also measured the mean Fulton index in the mice of 3 genotypes. The Fulton index was significantly higher in A_{2A}R KO mice compared to WT mice (mean $\pm$ SE: 26.44 ± 0.63 vs. $24.06 \pm 1.02\%$; fig. 2c, * $p < 0.05$, one way ANOVA followed by LSD test). Thus, A_{2A}R KO mice displayed hypertrophic RVs, consistent with a PAH phenotype.

A_{2A}R KO Mice Exhibited Hypertrophy of Pulmonary Resistance Vessels with Increased Wall Thickness and Area

Histological analysis revealed no pulmonary edema secondary to left ventricular dysfunction among the 3 genotypes. Moreover, lung sections with HE staining revealed no abnormalities such as infiltration of inflammatory cells, edema, fibrosis, hypercellularity or alteration/thickening of the alveolar septa in all genotypes. Thus, the PAH phenotype in A_{2A}R KO is not due to enhanced inflammation of lungs. To evaluate vascular remodeling of pulmonary resistance vessels, we determined the percentage of wall thickness and areas in pulmonary resistance vessels (25–100 μ m in diameter) in A_{2A}R KO mice and their WT littermates. As you can see from figure 3a and b, the mean percentage of wall thickness in the A_{2A}R KO mice (mean $\pm$ SE: $45.34 \pm 2.08\%$) was significantly higher than that of the WT mice ($38.28 \pm 2.91\%$). The mean percentage of wall thickness of the A_{2A}R+/- mice ($42.93 \pm 2.33\%$) displayed the intermediate value between the A_{2A}R KO and WT mice, indicating the gene-dose effect. Consistent with the wall thickness, the mean percentage of wall areas in A_{2A}R KO mice ($60.83 \pm 2.06\%$) was also significantly higher than in the WT mice ($54.51 \pm 3.30\%$; fig. 3c). Together, these results strongly suggest that hypertrophy of the pulmonary arterials is a result of PAH in A_{2A}R KO mice.

A_{2A}R KO Mice Exhibited Increased Expression of α -SMA and PCNA in Pulmonary Resistance Vessels

To determine the cellular basis for the increased thickness and area of pulmonary resistance vessels, we evalu-

Fig. 1. Expression of $A_{2A}R$ in small and large pulmonary arteries in WT and $A_{2A}R$ KO mice by fluorescence immunohistochemistry. $A_{2A}R$ immunoreactivity was determined in lung sections for WT and $A_{2A}R$ KO mice by fluorescence immunohistochemistry as described in Materials and Methods. Lung sections were co-stained with DAPI for total cells. **a–d** $A_{2A}R$ -positive cells in lung sections. **a1–d1** Nucleoli stained by DAPI. White arrow = pulmonary artery; red arrow = $A_{2A}R$ -positive cells in pulmonary artery; blue arrow = $A_{2A}R$ -positive cells in not pulmonary artery. $\times 200$.

ated the hyperplastic smooth muscularization in lung sections using an antibody against α -SMA (fig. 4). Small pulmonary arterials (25–100 μ m in diameter) were intensively stained with α -SMA immunoreactivity. Importantly, integrated optical density value of α -SMA in $A_{2A}R$ KO (mean $\pm$ SE: $1,099.30 \pm 87.03$) was significantly higher than in WT mice (840.00 ± 86.94 ; fig. 4). Thus,

increased thickness and area of pulmonary resistance vessels in $A_{2A}R$ KO mice are associated with enhanced expression or proliferation of SMCs. Furthermore, we determined cell proliferation in lung sections containing pulmonary resistance vessels. Increased PCNA+ cells were detected in all major cell types across entire pulmonary arterial walls of WT mice, including smooth muscle,

Fig. 2. $A_{2A}R$ KO mice exhibited elevation in RVSP and hypertrophy of RV. RVSP and hypertrophy measurements were made in adult $A_{2A}R$ KO and WT littermates as described in Materials and Methods. **a** Representative pictures of RVSP waves in WT and $A_{2A}R$ KO mice. **b** Mean RVSP (mm Hg) in WT ($n = 5$) and $A_{2A}R$ KO ($n = 7$) mice. $** p < 0.01$, $A_{2A}R$ KO vs. WT, one-way ANOVA, Dunnett's post hoc test. **c** Fulton index in WT and $A_{2A}R$ KO mice. WT, $n = 16$; $A_{2A}R$ KO, $n = 18$. $* p < 0.05$, $A_{2A}R$ KO vs. WT, one-way ANOVA, LSD test.

Fig. 3. $A_{2A}R$ KO mice exhibited hypertrophy of pulmonary resistance vessels with increased wall thickness and area. Pulmonary vascular wall thickness and wall areas in $A_{2A}R$ KO mice and WT littermates were assessed as described in Materials and Methods. **a** Representative photomicrograph of pulmonary vascular remodeling in lung. $\times 200$. **b** Percentage of wall thickness. **c** Wall areas in WT and $A_{2A}R$ KO mice. $* p < 0.05$, $A_{2A}R$ KO vs. WT, one-way ANOVA, LSD test, $n = 9$.

endothelium cell and fibroblasts (fig. 5). Importantly, the percentage of PCNA⁺ cells in A_{2A}R KO and A_{2A}R^{+/−} were significantly higher than in the WT mice (A_{2A}R KO $30.04 \pm 1.75\%$, WT $22.92 \pm 2.20\%$). Increased cell proliferation in A_{2A}R KO mice was observed in cell types across entire pulmonary arteries.

A_{2A}R KO Mice Exhibited Increased Activation and Proliferation of Endothelium Cells and Smooth Muscle as well as Enhanced Hyperplasia of Fibroblasts of Pulmonary Arteries at Ultrastructure Level

Compared with WT mice, the pulmonary arteries of the A_{2A}R KO mice showed swelling and hypertrophy of endothelial (fig. 6a) and SMCs (fig. 6b), with abundance of cytoplasm and of increased intracytoplasmic vesicles. The endothelium contained more Weibel-Palade bodies in A_{2A}R KO mice than WT mice. In the cytoplasm of SMCs, there were numerous filaments and dense bodies. It indicated the activation of endothelial cells and SMCs. Furthermore, while the internal and external elastic lam-

inae were unbroken in the 3 genotypes, more fibroblasts (fig. 6c) were seen outside the external elastic laminae of A_{2A}R KO mice than WT mice. There were also more clustered collagen fibers deposited in adventitia pulmonary arterial walls in A_{2A}R KO mice than WT mice. These ultrastructural changes are indicative of hyperplasia of fibroblasts. Together, ultrastructural analysis reveals increased activation and proliferation of endothelium cells and SMCs, hypertrophy of fibroblast and the increased deposition of collagen fibers in A_{2A}R KO mice, all contributing to the pulmonary vascular remodeling.

A_{2A}R KO Mice Displayed No Histological Features of Hypertensive Nephropathy

We also evaluated histological changes in renal arteries in A_{2A}R KO, A_{2A}R^{+/−} and WT mice (fig. 7). Capillary membranes of afferent glomerular arteriole were of normal thickness in the 3 genotypes. There is neither a hyaline nor a nodular-sclerotic appearance in lobules of the glomeruli in the mice of the 3 genotypes. α-SMA immu-

Fig. 5. $A_{2A}R$ KO mice exhibited increased PCNA-positive cells in pulmonary resistance vessels. Expression of α -SMA in pulmonary resistance vessels was determined by immunohistochemistry as described in Materials and Methods. **a, b** Representative photomicrographs of PCNA staining in lung. $\times 200$. **c** Quantitative analysis of PCNA-positive cells in lung sections of WT and $A_{2A}R$ KO mice. * $p < 0.05$, $A_{2A}R$ KO vs. WT, one-way ANOVA, LSD test, $n = 6-7$.

noreactivity in $A_{2A}R$ KO, $A_{2A}R$ +/- and WT mice were indistinguishable (fig. 7). Furthermore, no tubular atrophy, interstitial fibrosis or an inflammatory component were detected in renal sections of the 3 genotypes (fig. 7). Thus, there were no features of hypertension detected in the kidneys of the 3 genotypes. Specifically, histological features indicative of hypertensive nephropathy (for example, intimal fibroelastosis, hyaline arteriosclerosis and fibrinoid necrosis) were not seen in $A_{2A}R$ KO and WT mice.

Following Chronic Exposure to Hypoxia, $A_{2A}R$ KO Mice Exhibited Exacerbated Elevation in RVSP, Hypertrophy of Pulmonary Resistance Vessels and Increased Cell Proliferation in Pulmonary Resistance Vessels, Compared to WT Littermates

Lastly, we investigated whether $A_{2A}R$ gene deletion develops more severe PAH in a model of pulmonary arterial hypertension after exposure to hypoxia. A separate set of $A_{2A}R$ KO and WT littermates was exposed to a hy-

poxic chamber containing 10% oxygen in normobaric pressure for 2 weeks. Their RVSP were measured and histological changes of the lungs in these mice were examined. After exposure to hypoxia, both WT and $A_{2A}R$ KO mice increased RVSP compared to their corresponding groups exposed to normoxia (21% oxygen; fig. 8a). Interestingly, the increase in RVSP in $A_{2A}R$ KO mice compared to WT in normoxia was comparable to the increase in WT mice after exposure to hypoxia compared to WT mice in normoxia. Consistent with these hemodynamic changes, the mean percentage of wall area in WT and $A_{2A}R$ KO mice after exposure to hypoxia was significantly higher than that of their corresponding genotypes after exposure to normoxia (fig. 8b). Similarly, the increase in the wall area in $A_{2A}R$ KO mice compared to WT in normoxia was comparable to the increase in WT mice after exposure to hypoxia compared to WT mice in normoxia. However, two-way ANOVA analysis indicates that there was no interaction between genotype (WT vs. KO) and

Fig. 6. $A_{2A}R$ KO mice exhibited increased activation and proliferation of endothelium cells and smooth muscle as well as enhanced hyperplasia of fibroblasts of pulmonary arteries at ultrastructure level. Transmission electron micrographs of mouse pulmonary arteries: endothelial cell in pulmonary artery endomembrane layer (**a**), SMC in pulmonary artery tunica media layer (**b**) and collagen in pulmonary artery wall (**c**). Red arrow = cells in pulmonary artery (color only in online version); blue arrow = deposited collagen fibers; yellow arrow = Weibel-Palade bodies in endothelial cell. **a** $\times 12,000$. **b**, **c** $\times 10,000$.

oxygen condition (normoxia vs. hypoxia). These results indicate that pathological pulmonary hypertension by genetic deletion of $A_{2A}R$ in normoxia was equivalent to the increase in pulmonary hypertension induced by exposing WT mice to hypoxia for 2 weeks. The increase in pulmonary hypertension in $A_{2A}R$ KO mice under hypoxic condition seems to be an additive (not synergistic) effect of an $A_{2A}R$ KO effect under normal oxygen and a hypoxic effect in WT mice.

Furthermore, we also examined the effect of $A_{2A}R$ inactivation on cell proliferation (that is PCNA+ cells) in WT and $A_{2A}R$ KO mice after exposure to hypoxia. As expected, both $A_{2A}R$ KO and WT mice exhibited increased expression of PCNA in pulmonary resistance vessels after exposure to hypoxia (fig. 8c). Of note, the increase in WT mice exposure to hypoxia was clearly larger than the increase in $A_{2A}R$ KO mice compared to WT littermates under normal oxygen. However, two-way ANOVA analysis indicates that there was no interaction between the genotype and oxygen condition. The increase in PCNA+ cells in $A_{2A}R$ KO mice under hypoxic

condition seems to be an additive (not synergistic) effect of an $A_{2A}R$ KO effect under normal oxygen and a hypoxic effect in WT mice.

Discussions

Using the $A_{2A}R$ KO model, we have provided the first evidence for the critical contribution of $A_{2A}R$ to the development of PAH in mice. At the postnatal age of 14–16 weeks, $A_{2A}R$ KO mice exhibited characteristics of hemodynamic, histological and ultrastructural changes of PAH: (1) increased RVSP and the elevated Fulton index, as a result of increased right ventricular mass; (2) increased wall area and thickness and enhanced α -SMA immunoreactivity in pulmonary resistance vessels; (3) increased cellular proliferation in pulmonary resistance vessels as evident by increased PCNA-positive cells; (4) increased activation and hypertrophy SMCs and endothelium as well as collagen deposition in the adventitia of pulmonary arterial walls as revealed by electron micro-

Fig. 7. A_{2A}R KO mice displayed no histological features of hypertensive nephropathy. Expression of α -SMA in renal arteries was determined by immunohistochemistry as described in Materials and Methods. **a, b** Representative photomicrographs of α -SMA staining in kidney. $\times 200$. **c** Quantitative analysis of α -SMA-immunoreactivity (optical density) in WT and A_{2A}R KO mice. WT (n = 5), A_{2A}R KO (n = 7). $p > 0.05$, one-way ANOVA, LSD test.

scope examination; (5) following chronic exposure to hypoxia, A_{2A}R KO mice exhibited exacerbated elevation in RVSP, hypertrophy of pulmonary resistance vessels and increased cell proliferation in pulmonary resistance vessels, compared to WT littermates.

Spontaneous PAH and altered remodeling of pulmonary arterials at hemodynamic, histological and ultrastructural levels are supported by anatomical localization of A_{2A}Rs primarily in the vasculature to mediate vasodilation [9], and by functional demonstration that activation of A_{2A}R in endothelium cells mediates adenosine-induced vasodilation [9]. Adenosine can exert profound hypotensive effects by direct vasodilation [24], by stimulating nitric oxide release from vascular endothelial cells [25], and by attenuating the sympathetic nervous system [26], and by inhibiting the renin angiotensin system [27]. Thus, adenosine has been shown to elicit vascular smooth muscle relaxation in the pulmonary circulation and

hence has been proposed for the therapy of clinical and experimental pulmonary hypertension [28]. Our finding further argues that the adenosine effect is likely to be mediated by A_{2A}R in pulmonary vessels.

By contrast, hemodynamic and histological analyses revealed no features of hypertensive nephropathy in both genotypes and there was no significant difference in SBP, and left ventricular masses between A_{2A}R KO and WT mice. Furthermore, there was no histological feature of hypertensive nephropathy and the renal vasculature was indistinguishable among the 3 genotypes. This A_{2A}R KO phenotype is consistent with the pharmacological finding that intravenous infusion of adenosine decreases pulmonary arterial pressure without obvious change of systemic mean artery pressure [28]. This selective increase in pulmonary arterial hypertension is likely attributed to the fact that the normal pulmonary vasculature is a low-pressure system with less than one tenth the resistance to

flow observed in the systemic vascular bed [27], thus particular sensitive to adenosine-mediated hemodynamic and remodeling changes. While the hypotensive effect of A_{2A}R activation on SBP has been reproducibly demonstrated, pharmacological and genetic inactivation of A_{2A}R has produced mixed results on SBP. For example, chronic consumption of caffeine, a nonselective adenosine antagonist, has been associated with increased blood pressure in several epidemiological studies [28, 29]. However, many other epidemiological studies show that hypertensive effects were evident only at relatively high dose of caffeine (>250 mg/day) [30] and no association between regular caffeine consumption (<200 mg/kg) and hypertension was found [31, 32]. On the other hand, hypertensive phenotype was reported in A_{2A}R KO mice in CD1 strain (an outbreed strain) [17], but not in pure 129Sv or congenic C57BL/6 background or mixed 129Sv × C57BL/6 background [18, 19, 33].

Our finding of the lack of evidence for renal hypertensive phenotype and of indistinguishable mean SBP in the A_{2A}R KO and WT mice further substantiate the conclusion of the lack of A_{2A}R inactivation on SBP. The lack of renal damage caused by sustained systemic hypertension and the absence of the pulmonary edema secondary to left ventricular dysfunction in A_{2A}R KO mice suggest that left ventricular dysfunction and the increased resistance in systemic vascular bed are not the cause of PAH in A_{2A}R KO mice.

PAH phenotype was observed in the mature adult A_{2A}R KO mice (14–16 weeks old) so that the developmental effects of A_{2A}R gene deletion on the early (P3–P8, the rapid alveolar proliferation) and late postnatal (that is P42, increasing numbers of alveoli formed by growth processes) [34] were manifested at this mature adult age. It would be extremely interesting to examine the A_{2A}R KO and WT littermates at early as well as later develop-

ment stages. By defining hemodynamic and histological changes in these mice at early postnatal age (for example after 2–4 weeks), we may distinguish the pulmonary vascular remodeling as triggering factor of PAH or secondary response to PAH. On the other hand, characterization of hemodynamic and histological changes in more matured or aged mice (12- to 14-month-old mice) may uncover whether there is a progressive development of PAH and associated pathological changes in aged mice.

The exact mechanism underlying the development of PAH in A_{2A}R KO mice is not clear. Three major mechanisms including vasoconstriction, remodeling of pulmonary vessels wall and thrombosis are proposed to play critical roles in the development of PAH. Adenosine exerts several anti-vaso-occlusive effects, including inhibition of platelet aggregation [17, 35], diminished neutrophil adhesion to vascular endothelial cells and attenuation of neutrophil-induced endothelial cell damage [36]. However, the lack of the abnormalities such as infiltration of inflammatory cells, edema, fibrosis, hypercellularity or alteration/thickening of the alveolar septa in A_{2A}R KO mice strongly argues against the notion that pulmonary vaso-occlusion is unlikely the major contributor to the PAH phenotype in A_{2A}R KO mice. Furthermore, it was reported that variants of the genes for the A₁, A_{2A} and A_{2B} receptors do not appear to alter susceptibility to the disease of essential (systemic) hypertensives [37]. However, pulmonary artery vasoconstriction greatly contributes to a sustained elevation of pulmonary vascular resistance and pulmonary arterial pressure in patients with pulmonary hypertension [6]. It remains to be determined whether genetic variants or deletion of the A_{2A}R gene affect pulmonary vascular responsiveness, which might lead to vasoconstriction with pulmonary hypertension and progressive remodeling.

This abnormal pulmonary vascular remodeling contributes to the development of PAH in A_{2A}R KO mice. The pathological feature of PAH is characterized by an abnormal proliferation of endothelium and SMC, hypertrophy of SMC and abundant extracellular matrix deposition [38]. In support of this notion, we found that A_{2A}R KO mice displayed cells hyperplasia in pulmonary artery walls with increased immunoreactivity of α -SMA, as well as cell proliferation with increased PCNA+ cells in lung sections across entire pulmonary resistance vessels, indicating involvement of endothelium, smooth muscle and fibroblasts. Moreover, the enhanced activation and proliferation of endothelium cell and smooth muscle, and more importantly increased multiplication of collagen fiber and activation of fibroblasts were observed at ultra-

structural level in pulmonary artery walls of A_{2A}R KO mice compared to the WT littermates. These observations strongly suggested that A_{2A}Rs might affect pulmonary vascular remodeling, leading to PAH. Further experiments are warranted to dissect out the mechanism that A_{2A}Rs modulates the hyperplasia, proliferation and activation of various cells and the deposition of extracellular matrix in pulmonary artery walls.

Genetic mutations of BMP are found in approximately 30% of idiopathic PAH and 15% sporadic PAH. Gene deletion of vasoactive intestinal peptide [39] and a GTP-cyclohydrolase 1 [40] have been reported to develop moderate PAH with enhanced pulmonary vascular remodeling. This report represents the new example by which a single gene deletion produces selective PAH without affecting SBP. This finding invites epidemiological investigation into possible genetic association of variants of the A_{2A}R gene and PAH pathogenesis. Since the pulmonary vascular alteration in A_{2A}R KO mice closely resembles those pathologies of moderately severe idiopathic PAH, demonstration of the critical role of A_{2A}R in the development of PAH has important clinical implications for the treatment of PAH. Adenosine is a potent pulmonary vasodilator that has been used in therapy for clinical and experimental pulmonary hypertension [28]. Development of PAH in the absence of A_{2A}R suggests that the selective activation of A_{2A}Rs may be an important therapeutic strategy for the treatment of PAH.

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