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ORIGINAL ARTICLE

Sarpogrelate hydrochloride, a serotonin 5HT2A receptor antagonist, ameliorates the development of chronic hypoxic pulmonary hypertension in rats

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Abstract
Purpose The purpose of the present study was to determine if sarpogrelate hydrochloride (SPG), a serotonin 5HT2A receptor antagonist, prevented the development of chronic hypoxia-induced pulmonary hypertension (PH) and hypertensive pulmonary vascular remodeling.
Methods Forty-one male Sprague–Dawley rats were exposed to hypobaric hypoxia (380 mmHg, 10 % oxygen) or room air and administered 50 mg/kg SPG or vehicle by gavage once daily from day −2 to day 14. The mean pulmonary artery pressure (PAP) and right ventricular hypertrophy (RVH) were measured. Hypertensive pulmonary vascular remodelings were assessed morphometrically by light microscopy. Serotonin-induced contraction was determined in isolated pulmonary artery rings from 24 rats. In another set of rats, Western blotting, real-time polymerase chain reaction and immunofluorescent staining ($n = 9$) for lung tissue were performed.
Results Chronic hypoxia induced a rise in mean PAP and RVH, increased the percentage of muscularized arteries in peripheral pulmonary arteries and medial wall thickness in small muscular arteries, and potentiated serotonin-induced contraction, each of which was significantly ($p < 0.05$) ameliorated by SPG. Chronic hypoxia significantly increased the expression of endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (peNOS) protein levels, cyclic guanosine monophosphate, and matrix metalloproteinase-13 (MMP-13) mRNA levels in whole lung tissues. SPG increased peNOS expression in the immunofluorescent staining of peripheral pulmonary arteries from chronic hypoxic rats and decreased the MMP-13 mRNA in lung tissue in chronic hypoxic rats.
Conclusions The administration of SPG ameliorated the development of chronic hypoxic PH and hypertensive pulmonary vascular changes.

Keywords Pulmonary hypertension · Hypoxia · Nitric oxide · Sarpogrelate hydrochloride · Serotonin 2A receptor

Introduction

Chronic hypoxia causes pulmonary hypertension (PH), right ventricular hypertrophy (RVH), and hypertensive pulmonary vascular remodeling, leading to RV failure, impaired quality of life, and reduced survival [1, 2]. Patients with pulmonary parenchymal disease, sleep apnea, severe chronic obstructive pulmonary disease and people living at high altitudes develop PH, which is category III PH, i.e., lung disease/hypoxia-associated PH, according to the classification of PH [1]. In all conditions causing PH, vascular remodeling includes new muscularization of normally nonmuscular peripheral arteries and medial hypertrophy of

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muscular arteries [2–4]. Vasoconstriction also plays a role in the development of PH [5] and plasma levels of serotonin (5HT), a vasoconstrictor, are increased in patients and animals with PH [6, 7], which stimulates smooth muscle proliferation [1, 2]. Development of vascular remodeling in chronic hypoxia-induced PH was inhibited by 5HT2B [8], and 5HT1B/1D [9] receptor antagonist, but not by 5HT2A receptor antagonist, ketanserin, in mice [10].

Sarpogrelate hydrochloride (SPG), an another 5HT2A receptor antagonist, is a registered drug in Japan which improves vascular function in peripheral arterial disease [11] and in diseases associated with Raynaud's phenomenon [12], prevents recurrence in ischemic stroke patients [13], and improves coronary perfusion in coronary artery disease [14]. SPG reduced pulmonary artery pressure (PAP) in patients with systemic sclerosis having Raynaud's phenomenon by decreasing pulmonary arterial spasm [15]. However, the effect of SPG especially on chronic hypoxia-induced pulmonary vascular remodeling is to be determined. SPG attenuates endothelial nitric oxide synthase (eNOS) reduction in the lung from monocrotaline-induced PH [16] and in limb vascular endothelium from severe diabetic mice [17]. The purpose of the present study was to determine if SPG prevented the development of chronic hypoxic PH and pulmonary vascular remodeling. For eNOS and phosphorylated eNOS (peNOS) in lung tissues, Western blot analysis and immunohistochemistry were performed.

Methods

The Animal Experiment Committee of Mie University School of Medicine approved this study protocol. Male Sprague–Dawley rats weighing 180–200 g were obtained from SLC (Shizuoka, Japan). Rats were randomly assigned to one of four groups—normal rats kept in room air and treated with saline vehicle (Air/V, $n = 10$), rats kept in room air and treated with SPG (Air/SPG, $n = 9$), rats exposed to hypobaric hypoxia (air at 380 mmHg, 10 % oxygen) for 14 days and treated with saline vehicle (CH/V, $n = 12$), and rats exposed to hypobaric hypoxia for 14 days and treated with SPG (CH/SPG, $n = 10$). The administration of SPG (50 mg/kg daily by oral gavage) began 2 days before the hypoxic exposure period and continued until the last of the 14 days of hypoxic exposure in CH rats.

After 14 days, the rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.). A catheter (Silastic tubing, 0.31 mm inside diameter and 0.64 mm outside diameter) was inserted through the right external jugular vein into the pulmonary artery by a closed-chest technique, as previously described [4, 18–21]. Rats were then kept in room air. Forty-eight hours after catheterization, while the rats were fully conscious and quiet, mean pulmonary arterial pressure (mPAP) was measured with a physiological transducer and amplifier system (AP 620G, Nihon Kohden, Japan).

After measuring mPAP, lung sections were prepared for morphometric analysis of the vasculature using the barium injection method and were stained for elastin by the Van Gieson method, as previously reported [4, 18–21]. Light microscope slides were analyzed without prior knowledge of the treatment groups. All barium-filled arteries between 15 and 100 μm in diameter in each tissue section were examined at a magnification of ×400. The percentage of muscularized peripheral arteries at the alveolar wall and alveolar duct levels was calculated. For all muscular arteries between 50 and 200 μm in diameter (11–55 arteries were found per section), the wall thickness of the media (distance between the external and internal elastic laminae) was measured along the shortest curvature, and the percent medial wall thickness (%MWT) was calculated [4, 18–21]. The RV of the heart was dissected from the left ventricle plus the septum (LV + S) and was weighed separately. The ratio of RV/(LV + S) was calculated to evaluate RVH.

Vascular tension study

In the ring segments of the main pulmonary arteries isolated from the other sets of 24 rats with or without chronic hypoxic exposure, a cumulative concentration response curve to 5HT was obtained in the presence or absence of 10^{-6} mol/l SPG, as previously described [22, 23]. The contractions induced by 5HT were expressed as the percentage contraction of the maximal contraction induced by 70 mM potassium chloride (KCL).

Plasma 5HT, nitrate (NO_3^-), and lung tissue cyclic guanosine monophospate (cyclic GMP)

Concentrations of plasma 5-HT and NO_3^- were measured with high-performance liquid chromatography. Lung tissue cyclic GMP was measured using the cyclic GMP EIA Kit (Cayman Chemical Company, catalog no. 581021) in another set of 13 rats exposed to hypoxia with and without SPG for 14 days.

Western blotting for eNOS and peNOS

Western blotting for eNOS and peNOS in whole lung tissues [Air/V ($n = 4$), CH/V ($n = 4$), CH/SPG ($n = 5$)] was performed in rats exposed to hypoxia with and without SPG for 14 days. Lung tissues of approximately 80 mg were homogenized with 1.5 ml mammalian cell lysis kit (Sigma, MCL1-1KT, 081M4103), phosphatase inhibitor cocktail (Sigma, 071M4106), protease inhibitor cocktail (Sigma, 081M4109), and vanadate (Sigma, 016K0011),

centrifuged at 15,000 rpm at 4 °C for 20 min, and the supernatant was standardized to 3.5 mg/ml with a mammalian cell lysis kit. Standardized samples (500 μl) were mixed with 250 μl sample buffer solution (2ME+) (Wako, HSK9841) and then incubated at 95 °C for 5 min. Each 15 μl sample (total protein 34.5 μg) was subjected to SDS-PAGE on 8 % acrylamide gels and blotted onto a PVDF membrane. The PVDF was blocked in 5 % skimmed milk which was diluted in 0.1 % TBST. Three kinds of primary antibodies (anti-eNOS BD Transduction Laboratories G10296, 1:4,000 dilution; anti-peNOS Cell Signaling Ser-1177 #9571, 1:2,000 dilution; and anti-β-actin Sigma A5441, 1:200,000 dilution) were incubated in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo Co. Ltd., Japan) at 4 °C overnight and the secondary antibody (Amersham NA 931, 1:20,000 dilution) was incubated in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo Co. Ltd., Japan) for 1 h at room temperature. The PVDF membrane was incubated in Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, USA) for 5 min. Signals in the PVDF were captured digitally and densitometry was performed using Multi Gauge Ver. 3.0 (Fujifilm, Science Laboratory 2005, Japan).

cDNA preparation and Taqman real-time polymerase chain reaction (PCR) for matrix metalloproteinase-13 (MMP-13)

After extraction of total RNA from whole lung tissues using TRIzol reagent (Invitrogen, USA), 1 μg total RNA was synthesized to 20 μl cDNA with the ReverTra Ace (Toyobo Co. Ltd., Biochemical Operations Department, Osaka, Japan) and diluted to 200 μl. The Taqman Gene Expression Assay was used for analysis of MMP-13 (Rn01448194). cDNA samples (3 μl) were amplified in a total volume of 20 μl containing Taqman Gene Expression 2× Master Mix, 1 μl Taqman Gene Expression Assay, and 1 μl Rat ACTB (Rn00667869) Endogenous Control. Amplification was performed with StepOne Plus Real Time PCR Systems (Applied Biosystems). Relative quantification was performed with the comparative ΔΔCt method by normalization with β-actin mRNA.

Immunofluorescent staining of pulmonary arteries

Lung tissues were obtained from each group of rats [Air/V ($n = 3$), CH/V ($n = 3$), CH/SPG ($n = 3$)] and were fixed in Carnoy's solution (60 % methanol, 30 % chloroform, 10 % acetic acid) for 4 h. Sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. After blocking non-specific sites with 1 % skimmed milk in 0.01 M PBS for 30 min, sections were incubated with monoclonal primary antibody anti-eNOS (1:400, catalog No. 610297, BD Transduction Laboratories) and anti-peNOS (1:400, catalog No. 9571, Cell Signaling) overnight (4 °C). The sections were treated with goat anti-mouse IgG-Alexa594 (1:500, catalog No. A11032, Invitrogen) and goat anti-rabbit IgG-Alexa488 (1:500, catalog No. A11008, Invitrogen) for 2 h at room temperature. The stained sections were examined under an inverted laser scanning microscope (Fluoview FV1000, Olympus, Tokyo, Japan). To identify the immunonegative structures of endothelial cells in the blood vessels of the lung, neighboring sections were stained with hematoxylin and eosin.

Statistical analysis

Values are expressed as the mean ± SE. Statistical comparisons were analyzed using one-factor ANOVA. If a significant difference was found, Fisher's test was used to identify which groups were different. A level of $p < 0.05$ was accepted as significant.

Results

Weight gain and hematocrit

Chronic hypoxia significantly reduced body weight (Fig. 1). Chronic hypoxia significantly increased hematocrit from 40.96 ± 1.02 % in Air/V rats to 52.93 ± 0.76 % in CH/V rats ($p < 0.001$). SPG had no effect on these changes.

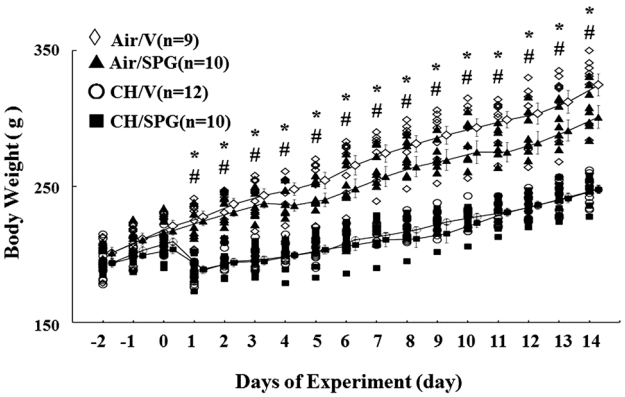

Fig. 1 Body weight. CH/V and CH/SPG rats lost weight during the first several days of hypoxic exposure, but regained it afterwards. In contrast, rats kept in ambient air gained weight steadily. From day 1 through to the last day, CH/V and CH/SPG rats had significantly lower body weights than those of Air/V and Air/SPG rats, respectively. Air/V rats exposed to ambient air and gavaged with vehicle, Air/SPG rats exposed to ambient air and gavaged with SPG, CH/V rats exposed to hypoxia (air at 380 mmHg) gavaged with vehicle, CH/SPG rats exposed to hypoxia for 14 days and gavaged with SPG. Values are mean ± SE, *n* number of rats, $^{*}p < 0.05$, Air/V rats versus CH/V rats. $^{\#}p < 0.05$, Air/SPG rats versus CH/SPG rats

mPAP and RVH

Chronic hypoxia significantly increased mPAP from 16.7 ± 0.4 mmHg in Air/V rats to 31.3 ± 0.7 mmHg in CH/V rats ($p < 0.001$). The prolonged administration of SPG significantly reduced this increase to 26.5 ± 0.8 mmHg in CH/SPG rats ($p < 0.001$) (Fig. 2a). The reduction in PAP by SPG was not due to a reduction in hematocrit values because no changes in these values were observed with and without SPG. Chronic hypoxia significantly increased RVH from 0.26 ± 0.01 in Air/V rats to 0.43 ± 0.02 in CH/V rats ($p < 0.001$). Prolonged administration of SPG significantly reduced this increase to 0.34 ± 0.01 in CH/SPG rats ($p < 0.001$) (Fig. 2b).

Vascular structural changes

Percentage of muscularized peripheral arteries

Chronic hypoxia significantly increased the percentage of muscularized arteries ($p < 0.001$) in peripheral pulmonary arteries. SPG significantly reduced this increase in arteries between 15 and 50 μm in external diameter ($p = 0.001$) (Fig. 3a).

Medial wall thickness

Chronic hypoxia significantly increased the %MWT ($p < 0.001$). SPG significantly reduced this increase in muscular arteries between 101 and 200 μm in external diameter ($p = 0.04$) (Fig. 3d).

Contractions induced by 5-HT in isolated pulmonary arteries

5HT induced similar contractions in the pulmonary artery rings of air rats with and without SPG. 5HT-induced contractions were significantly potentiated in the rings of CH/V rats ($p < 0.05$). Pretreatment with SPG significantly attenuated this chronic hypoxia-induced potentiation of contractions (Fig. 4).

Levels of plasma 5-HT, plasma nitrate (NO_3^-), and lung tissue cyclic GMP

There were no difference in plasma 5HT levels between Air/V rats and CH/V rats. SPG treatment increased the level in CH/SPG rats on hypoxia day 7 (Fig. 5a), but not on hypoxia day 14 (Fig. 5b). To detect the production of NO, its metabolite, nitrate (NO_3^-), and NO-induced substance, cyclic GMP, were measured. Chronic hypoxia significantly increased plasma levels of NO_3^- (Fig. 5c) and lung tissue cyclic GMP levels (Fig. 5d), suggesting an increase in NO production in chronic hypoxic rats. The effects of SPG on these values could not be detected.

Western blotting for eNOS and peNOS protein and Taqman real-time PCR for MMP-13 mRNA in whole lung tissue

eNOS (Fig. 6a) and peNOS (Fig. 6b) protein expression levels were significantly higher in the lung tissues of CH/V and CH/SPG rats than in the lung tissues of Air/V rats. Since chronic hypoxia increases MMP-13 expression in lung [24] and inhibition of protease activity ameliorates the pulmonary hypertensive vascular remodeling [25, 26], MMP-13 mRNA expression levels were measured. MMP-13 mRNA was significantly higher in CH/V rats than in Air/V rats and SPG significantly prevented MMP-13 mRNA upregulation (Fig. 7).

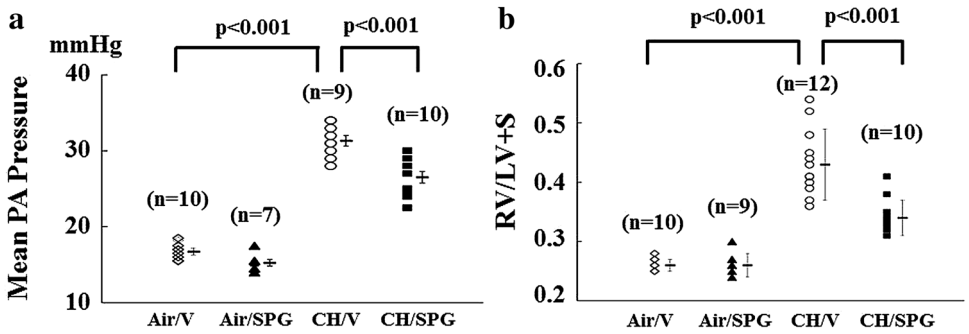

Fig. 2 mPAP and RVH. Mean pulmonary artery pressure (mPAP)—no significant difference in mPAP was observed between Air/V and Air/SPG rats. Chronic hypoxia significantly increased mPAP in CH/V rats. **a** Prolonged administration of SPG significantly reduced this increase in CH/SPG rats. Right ventricular hypertrophy (RVH)—chronic hypoxia increased RV/LV + S in CH/V rats. **b** Prolonged administration of SPG reduced this increase in CH/SPG rats. RV/LV + S ratio of the RV weight to the left ventricle plus the septum (LV + S) weight. Values are mean ± SE; *n* number of rats; see Fig. 1 for abbreviations

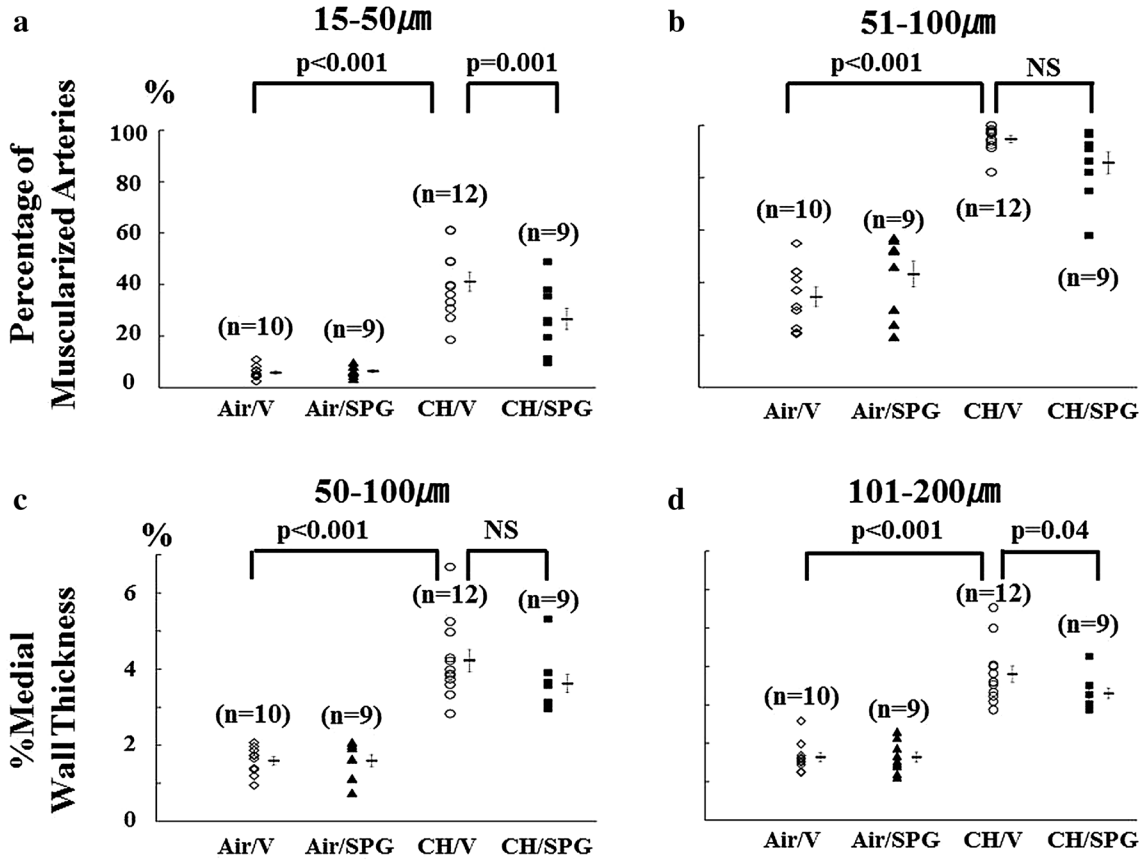

Fig. 3 Percentage of muscularized peripheral pulmonary arteries (**a**, **b**) and medial wall thickness (%MWT) (**c**, **d**). **a** 15–50 μm in external diameter. **b** 51–100 μm in external diameter. Chronic hypoxia significantly increased the percentage of muscularized arteries in peripheral pulmonary arteries between 15 and 50 μm in external diameter and in those between 51 and 100 μm in external diameter ($p < 0.001$). SPG significantly attenuated this increase in peripheral pulmonary arteries with an external diameter of 15–50 μm ($p = 0.001$). **c** 50–100 μm in external diameter. **d** 101–200 μm in external diameter. Chronic hypoxia significantly increased %MWT in small muscular arteries between 50 and 100 μm in external diameter and in those between 101 and 200 μm in external diameter ($p < 0.001$). SPG significantly attenuated this increase in arteries between 101 and 200 μm in external diameter ($p = 0.04$). Values are mean ± SE; *n* number of rats; see Fig. 1 for abbreviations

Immunofluorescent staining of eNOS and peNOS in pulmonary arteries

eNOS and peNOS expression were markedly detected in the endothelial cells of small muscular arteries in every group. peNOS immunofluorescent staining with the SPG treatment was higher in the muscular arteries of CH/SPG rats than in those of CH/V rats (Fig. 8).

Discussion

The long-term administration of SPG over 14 days of hypoxia resulted in a significant decrease in the severity of PH, which was associated with reductions in RVH, the percentage of muscularized arteries, and the medial wall thickness of muscular arteries. SPG increased peNOS expression in the immunofluorescent staining of peripheral pulmonary arteries from CH rats, decreased MMP-13 mRNA in lung tissue in chronic hypoxic rats, and reduced serotonin-induced contractions in isolated pulmonary arteries from CH PH rats.

Contractile responses to 5HT of rat pulmonary arterial rings were significantly increased in chronic hypoxic rats. SPG completely prevented this increase. Since SPG had no effect on 5HT-induced contraction in pulmonary arterial rings of control rats, 5HT2A receptor does not seem to mediate the contraction of normal pulmonary artery; however, after chronic hypoxic exposure, 5HT2A receptor-mediated contractions occur. This difference between normal and chronic hypoxic rats shows that 5HT2A receptor-mediated contraction occurs only in chronic hypoxic PH, leading to heightened contraction. Since plasma concentrations of 5HT were similar between air and hypoxic rats, the

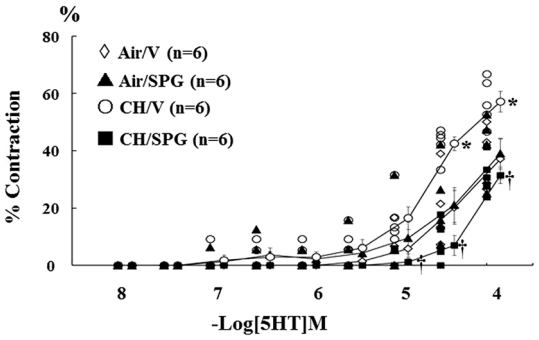

Fig. 4 Effects of SPG on contractions induced by 5HT. Responses to 70 mM KCL were taken as 100 %. Chronic hypoxia enhanced 5HT-induced contractions in isolated pulmonary arteries. Pretreatment with SPG attenuated contractions in pulmonary arteries from CH rats, although no effects were observed in air rats. Air/V pulmonary artery rings without SPG from rats exposed to ambient air, Air/SPG pulmonary artery rings with SPG from rats exposed to ambient air, CH/V pulmonary artery rings without SPG from rats exposed to hypoxia for 14 days, CH/SPG pulmonary artery rings with SPG from rats exposed to hypoxia for 14 days. Values are mean ± SE; *n* number of rats; $^{*}p < 0.05$ significantly different from Air/V rats; $^{\dagger}p < 0.05$ significantly different from CH/V rats

vascular tone induced by 5HT would have been increased in hypoxic rats. The attenuation of contraction results in the reduction of intravascular pressures on the vessel wall. Mechanical forces such as high pressure or flow induce vascular remodeling [27, 28]. Therefore, by reducing the pressure, SPG might prevent the development of new muscularization of peripheral pulmonary arteries and medial wall hypertrophy of muscular arteries.

An earlier study showed that another 5HT2A receptor antagonist, ketanserin, did not prevent the development of PH [10], which is different from the present study. Species (mice vs rats) and specificity to 5HT2A receptor might explain the difference. SPG has more selectivity towards 5HT2A receptor than ketanserin [29]. Ketanserin has other non-serotonergic binding sites [30].

Immunofluorescent staining of peNOS was higher in the peripheral pulmonary arteries of SPG-treated hypoxic rats than in untreated hypoxic rats, suggesting that SPG may upregulate the active form of eNOS, i.e., peNOS. Although SPG increased eNOS expression in diabetic mice [17], we could not detect the up-regulation of eNOS protein and

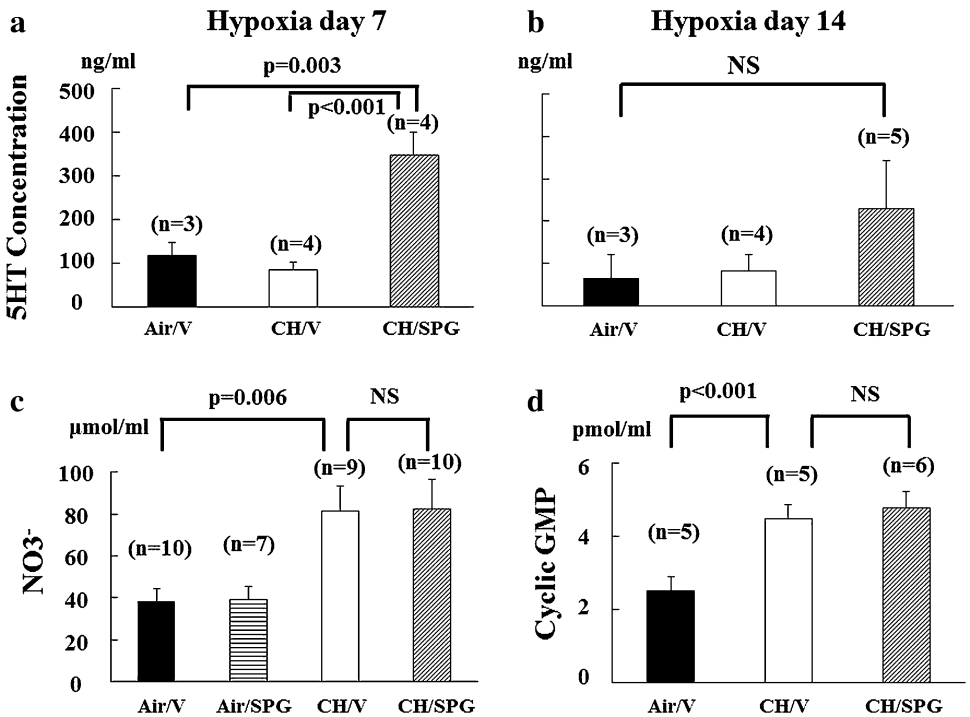

Fig. 5 Concentration of 5HT, NO_3^- and cyclic GMP. **a** plasma concentration of 5HT on hypoxia day 7. **b** plasma concentration of 5HT on hypoxia day 14. Plasma 5HT levels were similar in Air/V rats and CH/V rats. SPG significantly increased plasma 5HT levels in CH/SPG rats on hypoxia day 7 ($p < 0.05$). The selective blockade of 5HT receptors by SPG may prevent the binding of 5HT to 5HT receptors, thereby increasing receptor-unbound plasma 5HT levels in hypoxic rats. **c** plasma NO_3^-. The concentration of plasma NO_3^- was significantly higher in rats exposed to 14 days of hypobaric hypoxia than in normal rats. The SPG treatment had no effect on this change. **d** cyclic GMP in lung tissue. Chronic hypoxia significantly increased cyclic GMP levels in lung tissues. SPG treatment had no effect on this change. Air/V rats exposed to ambient air and gavaged with vehicle, CH/V rats exposed to hypoxia (air at 380 mmHg) for 14 days and gavaged with vehicle, CH/SPG rats exposed to hypoxia for 14 days and gavaged with SPG. Values are mean ± SE, *n* number of rats

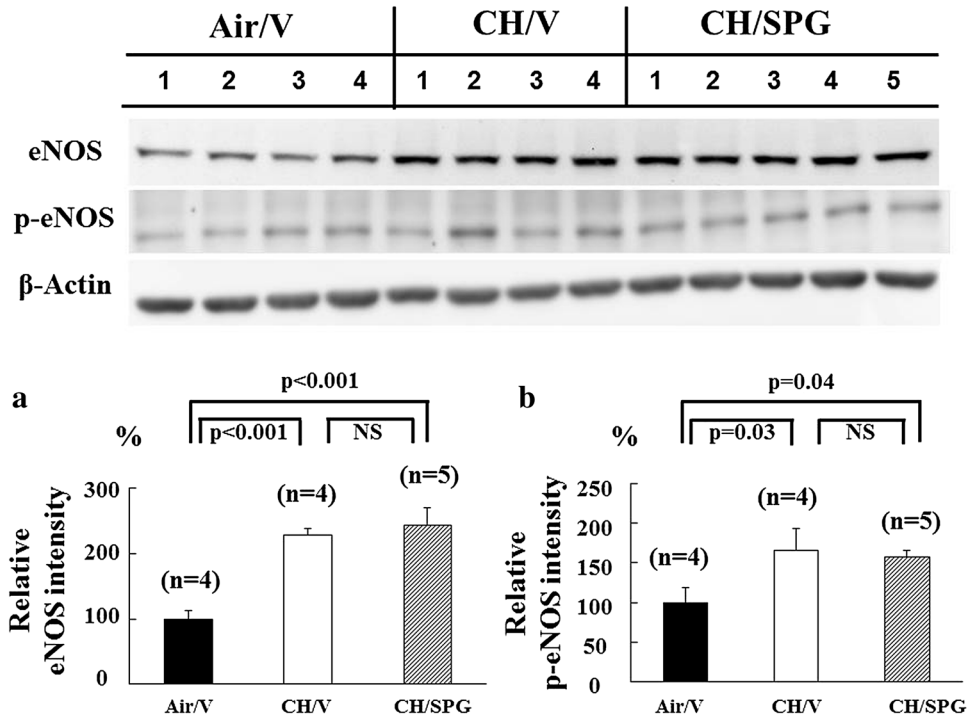

Fig. 6 Western blotting for eNOS and peNOS. Protein expression levels of eNOS were significantly higher in the lung tissues of CH/V and CH/SPG rats than in those of Air/V rats. SPG treatment had no effect on rats exposed to hypoxia (Fig. 5**a**). Air/V was standardized as 100 %. Protein expression levels of peNOS were significantly higher in the lung tissues of CH/V and CH/SPG than in those of Air/V rats. SPG had no effect on peNOS protein expression in rats exposed to chronic hypoxia (Fig. 5**b**). Air/V was standardized as 100 %. Values are the mean ± SE; *n* number of rats; see Fig. 5 for abbreviations

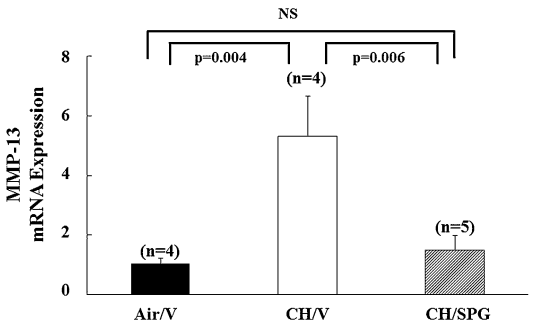

Fig. 7 Real-time PCR for MMP-13 mRNA. mRNA expression of MMP-13 significantly ($p = 0.004$) increased approximately five-fold in CH/V rats compared with Air/V rats. Treatment with SPG significantly ($p = 0.006$) reduced this increase in CH/SPG rats compared with CH/V rats. Air/V was standardized as 1. Values are mean ± SE; *n* number of rats; see Fig. 5 for abbreviations

cyclic GMP in hypoxic lung tissue in rats treated with SPG. Analysis of eNOS expression in whole lung tissues may not have been sensitive enough to detect subtle changes in peripheral tissues. Since a reduction in medial hypertrophy and new muscularization of peripheral pulmonary arteries were significant in SPG-treated rats, the upregulation of peNOS in these vessel walls may partly explain the effect of SPG on these arteries. Activation of the NO-cyclic GMP pathway prevented the development of experimental pulmonary hypertension, which included inhaled NO [27], the administration of the NO-precursor L-arginine [19], ANP gene transfection in the lung [21], and venous administration of eNOS-transfected smooth muscle cells [31] or bone marrow-derived endothelial-like progenitor cells [32]. Therefore, SPG induced-increase in local peNOS expression might partly contribute to the prevention of pulmonary vascular changes.

Chronic hypoxic exposure increased MMP-13 mRNA expression of lung tissue, which is consistent with previous studies showing increased vascular proteolytic activity in PH [25]. The origin of MMP-13 might be perivascular mast cells [24]. Since inhibition of matrix protease activity prevents the development of both monocrotaline- and chronic hypoxia-induced PH [25, 26], inhibition of MMP-13, matrix proteinase, in the present study might partly explain the preventable effect of SPG on chronic hypoxic PH.

It is necessary to examine the effects of SPG on other models of experimental PH. Earlier studies showed that SPG ameliorated the development of monocrotaline-induced PH [16, 33] and interleukin (IL)-6-induced pulmonary PH [34]. In inflammatory PH, intense inflammatory responses occur in which structural changes precede the rise in PAP [35]. The present results add chronic hypoxic PH to the group in which SPG ameliorates the development of PH. In chronic hypoxic PH, vasoconstriction precedes structural remodeling. Since a recent study showed that chronic hypoxic exposure exacerbated PH and pulmonary arteriopathy in animals with overexpression of IL-6, inflammatory mediators also modulate the disease process in chronic hypoxia-induced PH [36]. IL-6 causes mild PH and augments hypoxia-induced PH in mice [37]. Further studies would be necessary to determine if IL-6 is

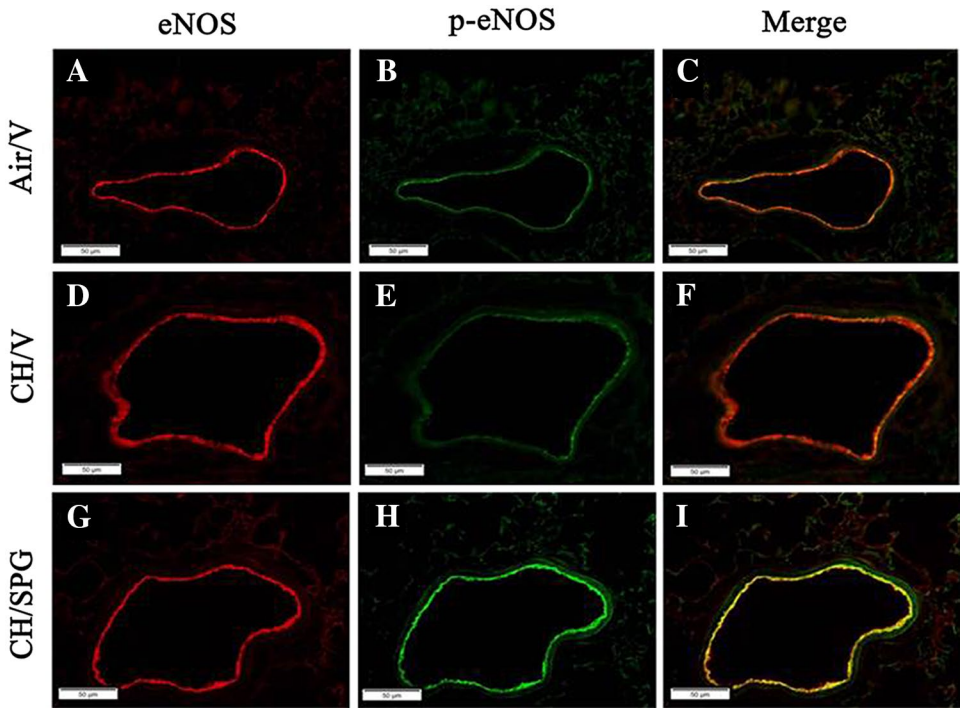

Fig. 8 Immunofluorescent staining of pulmonary arteries for eNOS and peNOS. Typical double immunofluorescent staining of pulmonary arteries in hypoxic rats on day 14 using antibodies against eNOS and peNOS. The first column, eNOS (*red*); the second column, peNOS (*green*); the third column, merged images. *Panels A–I*, muscular arteries between 101 and 200 μm in external diameter. eNOS and peNOS protein expression was detected in endothelial cells. peNOS expression levels with SPG treatment were higher in CH/SPG rats (*panel H*) than in Air/V (*panel B*) and CH/V rats (*panel E*). *Bar* 50 μm. See Fig. 5 for abbreviations (color figure online)

increased in the present model and if SPG would prevent this increase.

SPG is an approved drug in Japan and is indicated for ulceration in peripheral arterial disease and diseases associated with Raynaud's phenomenon such as pain and cold skin. Patients with collagen disease such as scleroderma, mixed connective tissue disease and systemic lupus erythematosis often have Raynaud's phenomenon and may develop PH. Although the dose of SPG in the present study is similar to other experimental PH models [16, 33, 34], it does not reflect the doses of SPG usually used in humans. There may be differences in bioavailability between rats and humans. Further investigation is necessary to explore the new indications of SPG for PH.

In summary, the administration of SPG ameliorated the development of chronic hypoxic PH and vascular remodeling. Attenuation of chronic hypoxia-induced potentiation of pulmonary arterial contraction, increased peNOS expression, and attenuation of chronic hypoxia-induced increase in MMP-13 mRNA levels were associated with administration of SPG.

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