Inhibition of Kinin B1 Receptors Attenuates Pulmonary Hypertension and Vascular Remodeling

Priya Murugesan, Tobias Hildebrandt, Christian Bernlöhr, Dongwon Lee, Gilson Khang, Henri Doods, Dongmei Wu

Abstract—This study examined whether the kinin B1 receptor is involved in the pathogenesis of pulmonary hypertension, and whether its inhibition could reduce inflammation, pulmonary hypertension, vascular remodeling, and right heart dysfunction. Male Wistar rats underwent left pneumonectomy. Seven days later, the rats were injected subcutaneously with monocrotaline (60 mg/kg). The rats were then randomly assigned to receive treatment with vehicle or with BI113823 (a selective B1 receptor antagonist, 30 mg/kg, twice per day) via oral gavage from the day of monocrotaline injection to day 28. By day 28, BI113823-treated rats had significantly lower mean pulmonary artery pressure, less right ventricular hypertrophy, and pulmonary arterial neointimal formation than that of the vehicle-treated rats. Real-time polymerase chain reaction revealed that there was a significant increase in mRNA expression of B1 receptors in the lungs of monocrotaline-challenged pneumonectomized rats. Treatment with BI113823 significantly reduced macrophage recruitment, as measured via bronchoalveolar lavage. It also markedly reduced CD-68 positive macrophages and proliferating cell nuclear antigen positive cells in the perivascular areas, reduced expression of inducible nitric oxide synthase, matrix metalloproteinase 2 and 9, and B1 receptors compared with measurements in vehicle-treated rats. These findings demonstrate that kinin B1 receptors represent a novel therapeutic target for pulmonary arterial hypertension. (Hypertension. 2015;66:906-912. DOI: 10.1161/HYPERTENSIONAHA.115.05338.) • Online Data Supplement

Key Words: hypertension, pulmonary hypertrophy, right ventricular inflammation, kinins, vascular remodeling, right heart hypertrophy, kinin B1 receptor antagonist

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by persistent increases in pulmonary arterial pressure. This increasing pressure is associated with perivascular inflammation, excessive pulmonary vascular proliferation, remodeling, and vasoconstriction. Ultimately, PAH can cause right heart failure and death.1-3 Despite new treatment options, PAH patients still face high mortality rates.1-3 Therefore, it is necessary to develop additional novel therapeutic approaches that target the various components of this multifactorial disease.1-3

Kinins are proinflammatory peptides that exert a variety of biological actions via stimulation of 2 pharmacologically distinct receptor subtypes, B1 and B2.4,5 The former are normally weakly expressed, but can be upregulated in the presence of cytokines and endotoxins or during tissue injury, whereas the latter are expressed constitutively.4,5 One important difference between the 2 subtypes is that the B2 is internalized rapidly and desensitizes, whereas kinin B1 receptor–induced responses are more persistent and suggesting the lack of the internalization of the B1 receptor–ligand complex.4,6 Thus, kinin B1 receptors represent a novel therapeutic target for chronic inflammatory diseases.3-6

Kinin B1 receptors are involved in diverse pathological processes, including inflammation, smooth muscle contraction, increased vascular permeability, edema, pain, cytokine and chemokine release, cell proliferation, and vascular and myocardial remodeling.5-9 In contrast to the B2 receptor–mediated relaxation to bradykinin, B1 receptors mediate vasoconstriction in cardiopulmonary vascular beds.5,10,11 We hypothesized that kinin B1 receptors may play an important role in the pathogenesis of PAH. With this same reasoning, B1 receptor antagonists may have therapeutic potential for treating PAH by reducing inflammation, inhibiting smooth muscle contraction, attenuating vascular and cardiac remodeling, and improving cardiac function.

The small molecule BI113823 is a newly developed, orally active, nonpeptide B1 receptor antagonist that exerts a potent anti-inflammatory effect with a favorable cardiovascular profile.7,9 This study examined the effects of kinin B1 receptor blockade with BI113823 in a well-established experimental model of monocrotaline-induced PAH and vascular remodeling in left pneumonectomized rats.12

Received February 10, 2015; first decision March 1, 2015; revision accepted August 4, 2015.
Department of BIN Fusion Technology, Chonbuk National University, Jeonju, South Korea (P.M., D.L., G.K., D.W.); Department of Respiratory Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany (T.H., C.B., H.D.); and Department of Research, Mount Sinai Medical Center, Miami Beach, FL (D.W.).
The online-only Data Supplement is available with this article at http://hyper.ahajournals.orglookup/supp;doi:10.1161/HYPERTENSIONAHA.115.05338/-/DC1.
Correspondence to Dongmei Wu, Department of BIN Fusion Technology, Chonbuk National University, Jeonju, South Korea. E-mail dongmeiwu@bellsouth.net
© 2015 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.115.05338
Methods

Detailed Methods are available in the online-only Data Supplement.

Monocrotaline-Induced Pulmonary Hypertension in Left Pneumonectomized Rats

On day 0, Male Wistar rats (250–300 g) were anesthetized with intramuscular injections of ketamine (80 mg/kg) and xylazine (10 mg/kg) before undergoing left pneumonectomy, as previously described by Faul et al.12 On day 7, rats were injected subcutaneously in the right hindlimb with monocrotaline (60 mg/kg, WAKO, Japan). These rats were then randomly assigned to receive treatment with vehicle (0.5% Natrosol+0.01% TWEEN 80, PO, BID, n=8) or with BI113823 (selective B1 receptor antagonist, 30 mg/kg, PO, BID, n=8) from the day of monocrotaline injection to day 28. All animals survived the entire experimental protocol.

Hemodynamic Measurement

On day 28, the right atrial pressure, right ventricular systolic blood pressure, and pulmonary arterial pressure were measured as previously described12 (for details, see the online-only Data Supplement).

Assessment of Pulmonary Inflammation

Bronchoalveolar lavage was collected and analyzed for inflammatory cell influx, total protein, tumor necrosis factor-α, and interleukin 1-β (IL-1β) levels.

Histological Analysis

Standard histopathologic procedures were used to prepare 5-μm-thick sections. The lung sections were stained with hematoxylin and eosin (Sigma, St. Louis, MO) and with Masson trichrome (American Master Tech Scientific, Inc, Lodi, CA) and were examined with light microscopy for morphological alterations.

Reverse-Transcription Polymerase Chain Reaction

Transcript levels of B1 and B2 receptors were assessed by quantitative reverse-transcription polymerase chain reaction as previously described.7

Immunohistochemical Imaging

Immunohistochemical analysis for endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), CD68, proliferating cell

Figure 1. BI113823 prevents the development of pulmonary arterial hypertension (PAH). Measurement of (A) mean pulmonary arterial pressure (mPAP), (B) right ventricular systolic pressure (RVSP), (C) ratio of right ventricular weight/left ventricular plus septum weight [RV/(LV+S)], (D) cardiac index (CI), (E) lung water content, and (F) lung mRNA expression of B1 and B2 receptors in sham control group rats (n=8), vehicle group rats [pneumonectomized rats received monocrotaline (60 mg/kg) and vehicle treatment; n=8], and BI113823 group rats (pneumonectomized rats received monocrotaline [60 mg/kg] and selective kinin B1 receptor antagonist BI113823 [30 mg/kg, PO, BID] treatment; n=8). All values are expressed as mean±SEM, n=6 to 8. *P<0.05 vs sham control; †P<0.05 vs vehicle.
nuclear antigen, matrix metalloproteinase (MMP)-9, MMP-2, and α-smooth muscle actin in lung sections were performed.

**Western Blot**

Western blot experiments were performed to determine protein expression of B1 receptor, eNOS, iNOS, CD68, proliferating cell nuclear antigen, MMP-9, and MMP-2 in lung tissues.

**Pulmonary Vascular Function and Smooth Muscle Cell Outgrowth**

Pulmonary artery relaxation responses to acetylcholine and sodium nitroprusside were measured using myograph methods. The effects of BI113823 on ex vivo pulmonary vascular smooth muscle cell migration and proliferation were performed.

**Statistical Analysis**

All data are reported as mean±SEM. Statistical differences were determined by ANOVA for repeated measures followed by Bonferroni’s post hoc test using GraphPad Prism 5. P values <0.05 were considered statistically significant differences.

**Results**

Three weeks after monocrotaline injection, compared with sham control rats, the vehicle-treated rats developed severe pulmonary hypertension with a significant increase in mean pulmonary artery pressure (↑ by 190%) and an increase in right ventricular systolic pressure (↑ by 140%; Figure 1A and 1B). While there was no significant changes in systemic blood pressure in all study groups (Table S1 in the online-only Data Supplement). As a consequence of increased pulmonary pressure, the vehicle-treated rats also developed significant right ventricular hypertrophy. The ratio of right ventricular weight/left ventricular and septum weight (RV/[LV+S]) increased by 90% in vehicle-treated rats when compared with that in sham control rats (Figure 1C). Over the course of the experiment, the vehicle-treated rats also exhibited a significant decrease in cardiac index and an increase in water content of their lungs when compared with sham control rats (Figure 1D and 1E). In contrast, treatment with BI113823 significantly reversed elevated mean pulmonary artery pressure (↓ by 52%), right ventricular systolic pressure (↓ by 39%), and RV/[LV+S] ratio (0.55 versus 0.38 in vehicle control), whereas increased cardiac index (↑ by 31%) compared with vehicle-treated rats (Figure 1A–1E). Reverse-transcription polymerase chain reaction revealed that kinin B1 and B2 receptors were expressed constitutively in rat pulmonary tissue. However, the mRNA expression of B1 receptors increased significantly in the monocrotaline-challenged pneumonectomized rats. In contrast, there was no significant change in the mRNA expression of B2 receptors in the pulmonary tissue (Figure 1F). These findings suggest that kinin B1 receptors play an important role in the development of pulmonary hypertension.

We quantitatively assessed the degree of pulmonary artery remodeling. Twenty-eight days after monocrotaline injection, the pulmonary arteries of the vehicle-treated rats were found to have extensive neointimal formation composed of α-smooth muscle actin–positive cells (Figure 2A). The pulmonary arteries from these vehicle-treated animals also had markedly increased medial wall thickness and increased

**Figure 2.** BI113823 prevents the development of neointimal formation in pulmonary arterial hypertension. **A**, Histological findings of the pulmonary arteries. **Top**, Hematoxylin and eosin (HE) staining. **Bottom**, Immunohistochemical staining of α-smooth muscle actin (αSMA). **B**, Medial wall thickness of pulmonary arteries and **(C)** vascular occlusion score of pulmonary arteries in each treatment group. All values are mean±SEM, n=7 to 8. *P<0.05 vs sham control; †P<0.05 vs vehicle.
vascular occlusion scores (Figure 2B and 2C). In contrast, the pulmonary arteries from animals treated with BI113823 had a significant decrease in medial wall thickness and in vascular occlusion scores. BI113823 also prevented the development of airway remodeling in rats (Figure S2). Furthermore, Masson trichrome staining showed marked decreases in collagen deposition in lung and right heart of animals treated with BI113823 compared with vehicle-treated controls (Figure 3A–3C).

Inflammatory cell recruitment is a key feature in the development of pulmonary artery hypertension. In this study, bronchoalveolar lavage samples from monocrotaline-challenged animals demonstrated a large increase in the number of macrophages and a small influx of neutrophils after monocrotaline challenge. In contrast, in rats treated with BI113823, the number of macrophages was only 40% of that of the vehicle-control animals (Figure 4A; Figure S1). Furthermore, protein content, tumor necrosis factor-α, and IL-1β levels in lavage were significantly lower in rats treated with BI113823 than those in vehicle controls (Figure 4B–4D).

Immunohistochemistry of vehicle-treated, monocrotaline-injured pulmonary tissue revealed that there was marked recruitment of CD-68 positive macrophages into perivascular areas, an increase in iNOS and a decrease in eNOS expression (Figure 5A and 5B). Immunohistochemistry also demonstrated substantial vascular cell proliferation in the thickened media layer of the PA. This layer was composed of proliferating cell nuclear antigen–positive cells in vehicle-treated monocrotaline-injured lung tissue (Figure 5C and 5D). In contrast, treatment with BI113823 attenuated the perivascular inflammation, smooth muscle cell proliferation, and nitrosative stress (Figure 5A–5D).

It also restored eNOS expression (Figure 5A and 5B). Levels of MMP-2 and MMP-9 protein were also greatly increased in vehicle-treated monocrotaline-injured lung tissue. Similarly, the expression of MMP-2 and MMP-9 was strongly suppressed by BI113823 treatment (Figure 5C and 5D). The reductions of CD-68, iNOS, proliferating cell nuclear antigen, MMP-2, MMP-9, and B1 receptor expression by BI113823 were confirmed by Western blot in lung tissues, but not eNOS (Figure S3). In addition, treatment with BI113823 improved both endothelium-dependent relaxations to acetylcholine and endothelium-independent relaxations to sodium nitroprusside in isolated pulmonary arteries (Figure S4) and attenuated growth factors and hypoxia-stimulated pulmonary artery smooth muscle cell migration and proliferation (Figure S5).

**Discussion**

The kinin B1 receptor mediates various inflammatory processes. This study examined whether the kinin B1 receptor is involved in the pathogenesis of PAH, and whether B1 receptor inhibition could attenuate inflammation and vascular remodeling, therefore preventing the development of PAH. Our data showed upregulation of B1 receptor mRNA expression in lung tissues from pneumonectomized rats challenged with monocrotaline. B1 receptor inhibition (with BI113823) protected lungs from developing PAH, vascular remodeling, and right ventricular hypertrophy. Treatment with BI113823 also reduced macrophage recruitment and cytokine production, inhibited vascular cell proliferation, and reduced expression of iNOS, MMP-2, and MMP-9 proteins.

Inflammation is a prominent pathological feature in PAH. Evidence from studies in animal models and in patients with PAH...
pulmonary hypertension suggests that inflammation contributes to the development of PAH.12–16 In lung biopsies from patients with PAH, infiltration of inflammatory cells, including macrophages and T and B lymphocytes, and dendritic cells is found in pulmonary perivascular spaces and around the plexiform lesions in PAH.17,18 Activation of macrophages induces the release of cytokines, such as tumor necrosis factor-α, IL-1β, and IL-6, which all are major contributing factors in the pathogenesis of PAH.19,20 In this study, bronchoalveolar lavage samples from monocrotaline-challenged left pneumonectomized rats demonstrated a large increase in the number of macrophages and a small influx of neutrophils after monocrotaline challenge. Furthermore, there was marked recruitment of CD-68 positive macrophages into perivascular areas of vehicle-treated, monocrotaline-injured pulmonary tissues. These findings further support an important role of inflammation in the pathogenesis of PAH, and that control of inflammation could be important for the prevention or treatment of PAH.

Kinin B1 receptors become upregulated following proinflammatory stimuli and then mediate diverse pathological processes.5–6 In patients with atheromatous disease, there is high kinin B1 receptor expression, but low kinin B2 receptor expression on foamy macrophages within thickened intimal plaques.21 Activation of B1 receptors stimulate leukocyte activation and chemotaxis, synthesis of cytokines and chemokines, and also increase vascular permeability.3,4,10 In this study, B1 receptor expression was upregulated in the lung tissues of monocrotaline-challenged pneumonectomized rats. Treatment with BI113823 significantly inhibited both chemokines- and hypoxia-induced migration and proliferation of rat pulmonary artery smooth muscle cells from pulmonary arterial explants. Furthermore, treatment with BI113823 inhibited the perivascular macrophages recruitment, reduced expression of iNOS, MMP-2, and MMP-9 proteins, and reduced pulmonary and cardiac fibrosis. Collectively, these effects prevented the development of PAH, vascular remodeling, and right ventricular hypertrophy.

Another key feature of PAH is vasoconstriction because of constrictive agents, such as endothelin-1, and an imbalance by a significant reduction in proinflammatory cytokine tumor necrosis factor-α, IL-1β release, and B1 receptor expression. These finding suggests that the BI113823-induced inhibition of the PAH and vascular remodeling is attributed to inhibition of macrophage recruitment and cytokine production. Pulmonary vascular cell proliferation and vascular remodeling is another important feature of PAH.1,2 In the media, smooth muscle cell proliferation, resulting in thickening of the vessel wall and muscularization of the arterioles, leads to both stiffening and vasoconstriction of the vessels.22 The increased pulmonary vascular resistance will consequently lead to right ventricular hypertrophy and dysfunction and premature death.1,2,22 Inflammatory processes play an important role in the cardiopulmonary remodeling of PAH.13–16 The degree of perivascular inflammation correlates with both vascular wall thickness and the mean pulmonary arterial pressure.18 Cytokines/chemokines and hypoxia are key players in the pathogenesis of PAH by mediating the excess cellular proliferation and pulmonary vascular remodeling.13–16 Human atherosclerotic lesions express high level of B1 receptors.21 Kinin B1 receptors mediate vascular remodeling and leukocyte invasion (monocytes) into the perivascular tissue in mice and rats.21 In this study, BI113823 significantly inhibited both chemokines- and hypoxia-induced migration and proliferation of rat pulmonary artery smooth muscle cells from pulmonary arterial explants. Furthermore, treatment with BI113823 inhibited the perivascular macrophages recruitment, reduced expression of iNOS, MMP-2, and MMP-9 proteins, and reduced pulmonary and cardiac fibrosis. Collectively, these effects prevented the development of PAH, vascular remodeling, and right ventricular hypertrophy.

Figure 4. In an experimental model of pulmonary arterial hypertension (PAH; induced by monocrotaline injection in pneumonectomized rats), BI113823 inhibits macrophage recruitment and cytokines production. A, Inflammatory cell count in bronchoalveolar lavage (BAL), (B) protein content, (C) tumor necrosis factor-α (TNF-α), and (D) interleulin-1β (IL-1β) in BAL of each treatment group. All values are mean±SEM, n=7 to 8. *P<0.05 vs sham control; †P<0.05 vs vehicle.
of vasodilators and vascular remodeling. Recent studies found little or no expression of NOS in the pulmonary vascular endothelium of patients with pulmonary hypertension. Various mediators, including cytokines, endotoxin, angiotensin II, and endothelin-1, induce transcriptional upregulation of kinin B1 receptors in vascular smooth muscle cells and subsequently increase vasoconstriction in cardiopulmonary vascular beds. B1 receptors have been shown to mediate strong contractile response to B1 receptor agonist des-Arg9-BK in pulmonary arteries in neonatal group B streptococcal sepsis in piglets and likely participate in the increase of pulmonary vascular resistance. Des-Arg9-BK decreases epicardial diameters in heart transplant patients. In isolated pig coronary arteries, we found that B1 receptors mediate strong, endothelium-independent coronary constriction in endotoxin-damaged arteries, but has no response in normal coronary arteries. Furthermore, B1 receptors mediate vasoconstriction in cardiopulmonary vascular beds by coupling to cyclooxygenases-2 and activation of thromboxane-prostanoid receptors. Kinin production increases during inflammation and inflammatory cells are sources of tissue kallikrein. Therefore, it is logical to presume that the local kinin production during inflammation can have pathologic significance in the pulmonary vascular tone and leading to PAH. In addition, endothelium-dependent relaxations to acetylcholine and endothelium-independent relaxations to sodium nitroprusside were impaired in vehicle-treated, monocrotaline-injured pulmonary arteries in this study. In contrast, treatment with BI113823 improved both endothelium-dependent and endothelium-independent relaxations in pulmonary arteries. Therefore, in addition to inhibition of inflammation and cardiopulmonary remodeling, kinin B1 receptor blockade may protect from PAH by regulating pulmonary vascular tone.

BI113823 is a small molecule orally active, nonpeptide B1 receptor antagonist that exerts a potent anti-inflammatory effect with a favorable cardiovascular profile. It exhibits high affinity (Ki) for both human and rat B1 receptors (5.3 and 13.3 nmol/L, respectively), whereas has no affinity for the B2 receptors (IC50>10,000 nmol/L). BI 113823 does not influence blood pressure in conscious rats. Furthermore, BI 113823 does not interfere with the blood pressure–lowering effects of lisinopril in spontaneously hypertensive rats after a 2-week treatment period. This study examined the effects of BI113823 in PAH in an experimental model of monocrotaline-induced PAH in pneumonectomized rats. This method produced severe pulmonary hypertension, right ventricular hypertrophy, and formation of obstructive intimal lesions in the peripheral pulmonary arterioles. However, this animal model may not precisely simulate all the forms of the pathobiology that might be present clinically in patients with idiopathic and heritable PAH.

**Perspectives**

Data from this study indicate that kinin B1 receptors play distinct roles in the development of PAH and vascular remodeling in an experimental rat model. Kinin B1 receptor inhibition may provide...
multiple protective factors against PAH, including reduction of inflammation and cytokine production, inhibition of smooth muscle constriction, reduction of vascular cell proliferation and vascular remodeling, and attenuation of right heart hypertrophy. Further studies are warranted to further elucidate the therapeutic potential of kinin B1 receptor antagonists for PAH.

Sources of Funding
This work was supported in part by Boehringer Ingelheim Pharma GmbH & Co. KG; the Brain Korea 21 PLUS Project, National Research Foundation; and the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012007351), Korea.

Disclosures
None.

References

Novelty and Significance
What is New?
• Data from this study reveal that kinin B1 receptors play an important role in the pathogenesis of pulmonary arterial hypertension.

What is Relevant?
• We report that the newly developed, small molecule, orally active, non-peptide B1 receptor antagonist, BI113823 attenuated pulmonary arterial hypertension, vascular remodeling, and right ventricular hypertrophy.

Treatment with BI113823 also reduced macrophage recruitment and cytokine production, inhibited vascular cell proliferation, reduced expression of inducible nitric oxide synthase, matrix metalloproteinase-2, and matrix metalloproteinase-9 proteins, and enhanced endothelial nitric oxide synthase expression.

Summary
These findings demonstrate that kinin B1 receptors represent a novel therapeutic target for pulmonary arterial hypertension.
Inhibition of Kinin B1 Receptors Attenuates Pulmonary Hypertension and Vascular Remodeling

Priya Murugesan, Tobias Hildebrandt, Christian Bernlöhr, Dongwon Lee, Gilson Khang, Henri Doods and Dongmei Wu

Hypertension. 2015;66:906-912; originally published online August 24, 2015;
doi: 10.1161/HYPERTENSIONAHA.115.05338

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/66/4/906

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/08/24/HYPERTENSIONAHA.115.05338.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Online supplements to:

Inhibition of Kinin B1 Receptors Attenuates Pulmonary Hypertension and Vascular Remodeling

Priya Murugesan\(^1\), Tobias Hildebrandt\(^2\), Christian Bernlöhr\(^2\), Dongwon Lee\(^1\), Gilson Khang\(^1\), Henri Doods\(^2\), and Dongmei Wu\(^{1, 3, *}\)

\(^1\)Department of BIN Fusion Technology, Chonbuk National University, Jeonju, South Korea;
\(^2\)Respiratory Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany;
\(^3\)Department of Research, Mount Sinai Medical Center, Miami Beach, FL, USA.

*Address correspondence to:

Prof. Dongmei Wu, Department of BIN Fusion Technology, Chonbuk National University, Korea, Telephone: 82-63-270-4755, Fax: 82-63-270-4254, E-mail: dongmeiwu@bellsouth.net
Supplementary Methods

These animal studies were approved by the Institutional Animal Care and Use Committee at Chonbuk National University, and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. A total of 24 male, 10-week-old Wistar rats, weighing between 250 and 300 grams were studied.

MCT-induced pulmonary hypertension in left pneumonectomized rats

On day 0, rats were anesthetized with intramuscular injections of ketamine (80 mg kg\(^{-1}\)) and xylazine (10 mg kg\(^{-1}\)) before undergoing left pneumonectomy, as previously described by Faul et al. (12). On day 7, rats were injected subcutaneously in the right hind limb with monocrotaline (MCT) (60 mg/kg, WAKO, Japan). These rats were then randomly assigned to receive treatment with vehicle (0.5% Natrosol + 0.01% TWEEN 80, p.o., b.i.d., n= 8), or with BI113823 (selective B1 receptor antagonist, 30 mg/kg, p.o., b.i.d., n= 8) from the day of MCT injection to day 28. The BI113823 dose was selected based on preclinical pharmacokinetic data. All animals survived the entire experimental protocol.

On day 28, rats were anesthetized using the same methods as previously, before a pulmonary arterial catheter was inserted through the right internal jugular vein. The right atrial pressure, right ventricular systolic blood pressure (RVSP), and pulmonary arterial pressure (PAP) were recorded using a Powerlab data acquisition system (ADInstruments Inc., CO). Next, another 2F miniaturized combined conductance catheter-micro-manometer (Model SPR-838, Millar instruments, Houston, TX) was inserted into the carotid artery to obtain the arterial pressure. Then, this catheter-micro-manometer was advanced into the left ventricle to record the left ventricular pressure and its first derivative (± dp/dp max).

Bronchoalveolar lavage (BAL) was collected through a 14-gauge angiocatheter. Lavages were collected twice after 2.5 ml of sterile PBS was perfused into the rat’s lung. A standard hemocytometer was used for BAL cell counting. Differential cell counts were performed on Giemsa-wright stained (Microscopy Hemacolor-Merck; Germany) cytospin preparations. The BAL protein concentration was determined using a Smart BCA Assay Kit (Intron Biotechnology Inc. South Korea). Enzyme immunoassay kits for mouse Interleukin 1-beta (IL-1β) (R & D Systems, Minneapolis, MN) and tumor necrosis factor (TNF)-α (BioLegend, San Diego, CA) were used to determine the concentrations of these mediators in BAL fluid. Lung and heart tissues were then collected and weighed. The wet-to-dry ratio was determined in one part of the lung. Liquid nitrogen was used to snap freeze one set of the tissues, while other tissues sets were fixed in buffered formalin for histopathological examination.

Histological analysis

Standard histopathological procedures were used to prepare 5-μm-thick sections. The sections were deparaffinized and stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA) and with Masson-Trichrome (American Master Tech Scientific, Inc. Lodi, CA, USA). The lungs
were examined with light microscopy for morphological alterations. All of the analysis was blinded. Parameters of vascular remodeling were performed using ImageJ software. The pulmonary arterial medial wall thickness was calculated as % wall thickness = (wall thickness × 2 / external diameter) × 100. The severity of neointimal formation was scored according to methods previously described (1). In this scoring system: 0 = the absence of neointimal lesion; 1 = less than 50% luminal occlusion; 2 = greater than 50% luminal occlusion. The average score of 50 vessels was obtained for each animal.

**Reverse-transcription polymerase chain reaction (RT-PCR)**

Transcript levels of B1 and B2 receptors were assessed by quantitative RT-PCR as previously described (2). Briefly, total RNA was extracted from lung tissues and cDNA was synthesized from 1 µg RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). For each RT-PCR reaction, 20 ng cDNA was used in a QuantiFast Probe PCR kit (Qiagen). The PCR primers from Applied Biosystems targeted the rat B1 receptor (Bdkrb1) (Gene Expression Assay Rn02064589_s1) and the rat B2 receptor (Bdkrb2) (Gene Expression Assay Rn00597384_m1). All data were normalized against RNA polymerase 2 (forward: GCAGGGCAGAGCGTGGGAG; reverse: CATTGGTATAATCAAACGGAACCTTC; probe: CTGGCTACACTTTAAGCCCTCTAATAAGC, FAM/TAMRA-labeled). Amplifications were performed in triplicate using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

**Western blot**

Western blot experiments were performed to determine protein expression of B1 receptor, (eNOS), iNOS, CD68, PCNA, (MMP)-9, and MMP-2 in lung tissues as previously described (2). Briefly, the lung protein extracts were separated by using SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with primary antibodies against B1 receptors, eNOS, iNOS, CD68, PCNA, MMP-9 (all from Santa Cruz Biotechnology, Santa Cruz, CA), and MMP-2 (Aviva systems biology, San Diego, CA), followed by incubation with HRP-conjugated secondary antibody. Immunoreactivity was detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Piscataway, NJ). Results were quantified using Image J software.

**Immunohistochemical analysis**

For IHC analysis, 5-µm-lung sections were deparaffinized, hydrated and incubated in 10mM sodium citrate buffer at 99°C for 20 minutes for antigen retrieval. Sections were incubated overnight with a primary antibody to one of the following antigens: endothelial nitric oxide synthase (eNOS), iNOS, CD68, proliferating cell nuclear antigen (PCNA), matrix metalloproteinase (MMP)-9 (all from Santa Cruz Biotechnology, Santa Cruz, CA), MMP-2 (Aviva systems biology, San Diego, CA), α-smooth muscle actin (α-SMA, Abcam, Cambridge, MA). Next, the section was incubated for 1 hour with FITC-labeled goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) or Alexa Fluor goat anti-rabbit IgG H &C
secondary antibody. Sections were counterstained with Ultra Cruz Mounting Medium with 4', 6-diamidino-2-phenylindole (DAPI; sc-24941, Santa Cruz Biotechnology) and cover slipped. Fluorescent images were taken using the Nikon Eclipse TE2000-U fluorescence microscope (Nikon Corp., Tokyo, Japan) and a Nikon LWD 0.52 digital camera. Fluorescent intensity was quantified using Image Pro Premier 9.1 software.

Relaxation responses of the pulmonary arteries

Pulmonary arteries were isolated and cleaned of all fat and connective tissue. Segments (~2 mm) of artery rings were mounted under the optimum resting tension (~0.7 g) in a 8 mL small vessel wire myograph chamber, filled with Krebs-Ringer solution [in mmol/l: NaCl 120; KCl 4.76; MgSO₄ 1.18; NaHCO₃ 25; NaH₂PO₄ 1.18; glucose 5.5; CaCl₂ 1.25] at 37°C and aerated with 95% O₂ and 5% CO₂ to maintain PO₂ at ~ 40 mmHg. After 60 minutes of equilibration, each preparation was exposed twice to 60 mmol/L potassium at a 30 minutes interval. Subsequently, relaxations in response to acetylcholine (ACh) and to sodium nitroprusside (SNP) were examined in U46619-contracted rings.

Pulmonary arterial smooth muscle cell (PASMC) outgrowth

Rat pulmonary arteries were carefully removed, suspended in sterile phosphate-buffered saline, and prepared for tissue culture under sterile conditions. Explant cultures were prepared as previously described (6). Briefly, the endothelium was removed by gently rubbing. The artery was then cut into 2-mm² pieces that were explanted in 12 well culture plates. The explants were cultured with Smooth Muscle Cell Growth Medium 2 (PromoCell GmbH) containing 10% fetal bovine serum and were kept in a humidified atmosphere of 5% CO₂/95% air at 37°C. For immunolabeling of PASMCs with α-smooth muscle actin, rat PASMCs were washed in PBS and then fixed in acetone (~20°C) for 10 minutes. Fixed cells were subjected to immunostaining with α-smooth muscle actin as above described.

In the first set of experiments, the effects of BI113823 (0, 0.01, 0.1, 1, and 10 µM) on PASMCs migration and proliferation was determined in cultures stimulated by growth factors: Smooth Muscle Cell Growth Medium 2 supplemented with 10% fetal bovine serum, 0.5 ng / ml human recombinant epidermal growth factor, 2 ng / ml human recombinant fibroblast growth factor, and 5 µg / ml recombinant human Insulin. Medium was changed every two days. PASMCs were counted after 7 days of culture. Triplicate wells were used for each concentration. The same experiment was repeated three times. In the second set of experiments, the effects of BI113823 on PASMCs migration and proliferation were determined in cultures stimulated by hypoxia as described previously (7). Briefly, the explants were cultured with Smooth Muscle Cell Growth Medium 2 (PromoCell GmbH) containing 10% fetal bovine serum (without growth factors) either in normoxic, or in hypoxic conditions. The hypoxic environment was created by placing a cell culture plates in a sealed chamber flushed with N2 and with one Anaero Pack-MicroAero (Mitsubishi Gas Chemical Co, Tokyo, Japan) which absorbs oxygen and generates carbon
dioxide. Medium was changed every two days. PASMCs were counted after 5 days of culture. Triplicate wells were used for each concentration. The same experiment was repeated three times.

Supplementary Results

1. Hemodynamic changes

Hemodynamic parameters are shown in table S1. There were no significant changes in mean blood pressure (mBP), heart rate (HR) or left ventricle systolic pressure (LVP) in any of the study groups. Vehicle-treated control animals showed elevated left ventricle end-diastolic pressure (LVEDP) compared to vehicle controls. LVEDP elevation was significantly attenuated in BI113823 treated animals (table S1).

2. Bronchoalveolar lavage (BAL)

The majority of cells that found in bronchoalveolar lavage are macrophages as determined by cell morphology (H&E staining, figure S1A) and by immunostaining with CD68 (figure S1B).

3. Pathological changes in airway

The small airway pathological changes were also assessed by HE staining (Figure 2A) and a quantitative evaluation of the thickness of the small airway was determined by WAt/Pbm (figure S2B). The small airways of the vehicle-treated rats were found to have extensive remodeling. In contrast, animals treated with BI113823 showed a marked decrease in airway remodeling as evidenced by a significant decrease in WAt/Pbm ratio (figure S2B).

There is mounting evidence that PAH, asthma and chronic obstructive pulmonary disease (COPD) share important pathological features, including inflammation, smooth muscle contraction and remodeling (3, 4). There is also a high incidence of mild to moderate PAH prevalence, reaching to 50% in advanced chronic obstructive COPD (5). These shared pathological features suggest possible common underlying mechanism among PAH/asthma/COPD. In the present study, MCT-treated pneumonectomized rats showed marked airway remodeling. Treatment with BI113823 significantly attenuated airway remodeling compared to vehicle treated rats. These findings suggest that B1 receptor may represent one common underlying mechanism among chronic pulmonary inflammatory diseases such as PAH, asthma and COPD. It warrants further investigation.

4. Western blot

Western blot analysis showed that treatment with BI113823 significantly attenuated the expression of CD-68, iNOS, PCNA, MMP-2, MMP-9 and B1 receptors in lung tissues compared to vehicle controls (Figure S3). In contrast, there was marked increase in eNOS expression in lung tissues of vehicle and drug treated animals compared to control animals without undergo left pneumonecctomy. This increase in eNOS expression may possibly reflect the compensative
remodeling and angiogenesis resulting from left pneumonectomy. Further study is warranted by using a different animal model of pulmonary hypertension.

5. Functional responses of the pulmonary arteries

Endothelium-dependent relaxations to acetylcholine (1-10 µM) and endothelium-independent relaxations to sodium nitroprusside (0.03-0.3 µM) were significantly impaired in pulmonary arteries of the vehicle treated animals compared to normal control rats (figure S4A-B). In contrast, relaxations to acetylcholine and sodium nitroprusside were significantly improved in animals treated with BI113823, compared to vehicle treated animals (figure S4A-B).

6. Pulmonary vascular smooth muscle cell outgrowth

Abnormal growth of pulmonary artery smooth muscle cells (PASMCs) is a major pathological condition in pulmonary arterial hypertension (1). In the present study, rat PASMCs began to migrate and proliferate from explants after 2-3 days in culture (Figure S5A-B). Cells were counted after 7 days in culture. BI113823 (0.1–10 µ mol/L) significantly inhibited growth factor stimulated migration and proliferation of rat PASMCs (Figure S5C). Hypoxia caused significant increase in PASMC migration and proliferation compared to normoxia (Figure S5D). BI113823 (0.01–10 µ mol/L) also significantly inhibited hypoxia stimulated migration and proliferation of rat PASMCs (Figure S5D).

Supplementary References

Supplementary Table

**Table S1.** Hemodynamic parameter changes in rats three weeks after MCT injection. All values are mean ± SEM, n=7. *p < 0.05 vs. control; †p<0.05 vs. vehicle.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham control</th>
<th>Vehicle</th>
<th>BI 113823</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>262 ± 12</td>
<td>274 ± 15</td>
<td>265 ± 9</td>
</tr>
<tr>
<td>mBP (mmHg)</td>
<td>85.4 ± 7.3</td>
<td>82.4 ± 8.3</td>
<td>87.2 ± 8.6</td>
</tr>
<tr>
<td>LVP (mmHg)</td>
<td>98.4 ± 8.1</td>
<td>91.2 ± 10.3</td>
<td>101.2 ± 9.7</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.4 ± 0.2</td>
<td>7.5 ± 0.5*</td>
<td>3.7 ± 0.3†</td>
</tr>
</tbody>
</table>
Supplementary Figures

Figure S1.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Vehicle</th>
<th>BI 113823</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Vehicle</th>
<th>BI113823</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>CD-68</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>DAPI</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Merged</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure S1.** (A) Representative Giemsa-wright staining of immune cells in BAL fluid in each treatment group. (B) Immunohistochemical staining for CD-68 (as a marker for the CD-68 positive macrophages) in BAL cells in each treatment group.
Figure S2. BI113823 prevents the development of airway remodeling in rats. (A) Representative hematoxylin and eosin staining of the airways; (B) the area of airway wall (WAt) / the basement membrane perimeter (Pbm) of the airways in each treatment group. All values are mean ± SEM, n = 7-8. *p < 0.05 vs. Control, †p < 0.05 vs. vehicle.

Figure S3.
Figure S3. Western blots of the expression of CD-68, iNOS, PCNA, MMP-2, MMP-9, eNOS, B1 receptors (B1R) and β-actin in lung tissues. (A) Representative immunoblots of CD-68, iNOS, PCNA, MMP-2, MMP-9, eNOS, B1 receptors and β-actin. (B) Mean densitometric analysis of immunoblots. All values are mean ± SEM. N= 5-6. *p < 0.05 vs. the sham control group, †p < 0.05 vs. the vehicle group.
Figure S4. Endothelium-dependent relaxations to acetylcholine and endothelium-independent relaxations to sodium nitroprusside in the isolated rat pulmonary arteries in each treatment group. All values are mean ± SEM, n = 7. *p < 0.05 vs. control group; †p < 0.05 vs. the vehicle group.
Figure S5. Effect of BI113823 on rat pulmonary artery smooth muscle cell (PASMC) migration and proliferation. (A) Representative photographs of rat PASMC outgrowth at day 6. (B) Immunohistochemical staining for αSMA (as a marker for the smooth muscle cells). (C) BI113823 concentration-dependently inhibited pulmonary artery SMC migration and proliferation stimulated by growth factors. (D) BI113823 concentration-dependently inhibited pulmonary artery SMC migration and proliferation stimulated by hypoxia. All values are mean ± SEM, n = 6-8. *p < 0.05 vs. Control.