Activation of Soluble Guanylate Cyclase Reverses Experimental Pulmonary Hypertension and Vascular Remodeling

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Background—Severe pulmonary hypertension is a disabling disease with high mortality, characterized by pulmonary vascular remodeling and right heart hypertrophy. Using wild-type and homozygous endothelial nitric oxide synthase (NOS3−/−) knockout mice with pulmonary hypertension induced by chronic hypoxia and rats with monocrotaline-induced pulmonary hypertension, we examined whether the soluble guanylate cyclase (sGC) stimulator Bay41-2272 or the sGC activator Bay58-2667 could reverse pulmonary vascular remodeling.

Methods and Results—Both Bay41-2272 and Bay58-2667 dose-dependently inhibited the pressor response of acute hypoxia in the isolated perfused lung system. When wild-type (NOS3+/+) or NOS3−/− mice were housed under 10% oxygen conditions for 21 or 35 days, both strains developed pulmonary hypertension, right heart hypertrophy, and pulmonary vascular remodeling, demonstrated by an increase in fully muscularized peripheral pulmonary arteries. Treatment of wild-type mice with the activator of sGC, Bay58-2667 (10 mg/kg per day), or the stimulator of sGC, Bay41-2272 (10 mg/kg per day), after full establishment of pulmonary hypertension from day 21 to day 35 significantly reduced pulmonary hypertension, right ventricular hypertrophy, and structural remodeling of the lung vasculature. In contrast, only minor efficacy of chronic sGC activator therapies was noted in NOS3−/− mice. In monocrotaline-injected rats with established severe pulmonary hypertension, both compounds significantly reversed hemodynamic and structural changes.

Conclusions—Activation of sGC reverses hemodynamic and structural changes associated with monocrotaline- and chronic hypoxia-induced experimental pulmonary hypertension. This effect is partially dependent on endogenous nitric oxide generated by NOS3. (Circulation. 2006;113:286-295.)

Key Words: cardiovascular diseases ▪ hypertension, pulmonary ▪ muscle, smooth ▪ nitric oxide ▪ pharmacology

Pulmonary arterial hypertension is characterized by lung vascular remodeling, high pulmonary blood pressure, and right ventricular hypertrophy. Hypoxia is considered a major factor in the pathogenesis of pulmonary hypertension, eg, in pulmonary obstructive and restrictive diseases and at high altitude. Acute hypoxia causes a selective pulmonary arteriolar vasoconstriction and increases pulmonary blood pressure, whereas exposure to chronic hypoxia induces structural and functional changes in the pulmonary arterial bed.1,2 These changes include proliferation and migration of smooth muscle cells as well as an increased accumulation of extracellular matrix. Imbalances in vasodilatory and vasoconstrictive forces have been implicated in both the predominance of increased vasomotor tone and the chronic remodeling of resistance vessels. Nitric oxide (NO) synthesized by endothelial NO synthase (eNOS, NOS3) is a potent vasodilator and is considered to play an important role in regulating pulmonary vascular tone. The downstream effector of NO is soluble guanylate cyclase (sGC), which synthesizes the second messenger cyclic guanosine monophosphate (cGMP).

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Although impairment of the endothelium-dependent regulation of pulmonary vascular tone is reported consistently, the analysis of the role of sGC in chronic hypoxia-induced pulmonary arterial hypertension has yielded conflicting data, with both increase and decrease of sGC protein expression described.3–5 Potential therapeutic potential has been reported for YC-1, which acts as a “NO sensitizer,” greatly enhancing the sensitivity of sGC toward this soluble agent.6,7 YC-1 increases cGMP in smooth muscle cells and induces a dose-dependent vasodilation of endothelium-denuded rat aortic rings.8–10 Furthermore, YC-1 has been shown to inhibit the adhesion and aggregation of platelets.11–13
Recently, the compound Bay41-2272, which stimulates sGC directly and enhances the sensitivity of sGC to NO, was shown to be a systemic and pulmonary vasodilator.\(^{14,15}\) Furthermore, it augments the vasodilative response to inhaled NO in acute pulmonary hypertension in lambs.\(^{16}\) Whereas Bay41-2272 activates sGC in its native form, another compound, Bay58-2667, has recently been shown to activate sGC even in its oxidized or heme-free form and independently of NO.\(^{17}\)

The aim of this study was to test the hypothesis that both compounds reverse pulmonary vascular remodeling in chronic experimental pulmonary hypertension in mice and rats. Chronic hypoxia was applied to induce pulmonary hypertension in mice, and the injection of the plant alkaloid monocrotaline was used in rats to induce a more aggressive form of pulmonary hypertension. To investigate the role of endogenous NO in this putative antiremodeling pathway, we tested this hypothesis in both wild-type and eNOS (NOS3) knockout mice with hypoxia-induced pulmonary hypertension.

**Methods**

**Animals**

Adult male Sprague-Dawley rats (350 to 400 g body wt) and C57Bl/6J and NOS3/−/− (Nos3tm1Unc, Jackson Laboratories) mice were obtained from Charles River Laboratories. Animals were housed under controlled temperature (≈22°C) and lighting (12/12-hour light/dark cycle), with free access to food and water. All experiments were performed according to the institutional guidelines that comply with national and international regulations.

**Hemodynamics**

The animals were anesthetized with ketamine/xylazine (intrapertorineally) and placed on a heating pad to maintain the body temperature in the physiological range. They were tracheostomized and artificially ventilated with 10 mL/kg body wt (SAR830A/P, ITT). Inspiratory oxygen (FiO\(_2\)) was set at 0.5, and a positive end-expiratory pressure of 1.0 cm H\(_2\)O was used throughout. The systemic arterial pressure (SAP) was monitored by cannulating the left carotid artery with a polyethylene cannula connected to a fluid-filled force transducer (Braun). The right jugular vein was used for catheterization of the right ventricle with a custom-made silicone catheter. The transducers were calibrated before every measurement. For a continuous measurement of SAP and heart rate, radiotelemetric sensors were implanted into anesthetized mice (Dataquest A.R.T. 2.1; Data Science Inc). The system comprises a fluid-filled sensing catheter (5 cm long, external diameter 0.7 mm, internal diameter 0.25 mm; model TA11PA) connected to a transmitter that signals to a remote receiver (model RPC-1) and a data exchange matrix connected to a computer. After surgery, mice were allowed to recover for 3 days. The SAP stabilized in the first 24 hours. None of the animals manifested signs of inflammation or infection.

**Isolated Perfused Mouse Lung**

The effects of Bay41-2272 and Bay58-2667 on acute hypoxic pulmonary vasoconstriction were examined in isolated ventilated perfused mouse lungs.\(^{18}\) Briefly, C57Bl/6J mice weighing 22 to 22 g were anesthetized as described above. Tracheostomy was performed, and the animals were ventilated with room air with the use of a Minivent 845 (Hugo Sachs Electronics, Harvard Apparatus GmbH) respirator. After midsternal thoracotomy, catheters were placed into the pulmonary artery and the left ventricle. The technique of successive hypoxic maneuvers in buffer-perfused lungs has been described previously.\(^{19}\) Sequential hypoxic maneuvers of 10-minute duration interrupted by 15-minute periods of normoxia were performed. The effects of the various pharmacological agents on pressure responses provoked by alveolar hypoxia (1% O\(_2\)) were determined within such a sequence of repetitive hypoxic maneuvers. Each agent was added to the buffer fluid 5 minutes before a hypoxic challenge, with the addition starting after the second hypoxic maneuver was accomplished. Cumulative dose-effect curves were established by addition of either Bay41-2272 or Bay58-2667 in the reservoir (dose range, 0.001 to 10 μmol/L).

**Radiotelemetry**

For a continuous measurement of SAP and heart rate, radiotelemetric sensors were implanted into anesthetized mice (Dataquest A.R.T. 2.1; Data Science Inc). The system comprises a fluid-filled sensing catheter (5 cm long, external diameter 0.7 mm, internal diameter 0.25 mm; model TA11PA) connected to a transmitter that signals to a remote receiver (model RPC-1) and a data exchange matrix connected to a computer. After surgery, mice were allowed to recover for 3 days. The SAP stabilized in the first 24 hours. None of the animals manifested signs of inflammation or infection.

**Hypoxia and Treatment With Bay41-2272 and Bay58-2667**

Pulmonary hypertension was induced by exposure to hypoxia (10% inspired O\(_2\) fraction) in a normobaric chamber as described previously.\(^{16}\) Mice were exposed to hypoxia for 21 or 35 days in a hypoxic normobaric chamber (n=10 each). Control animals were placed in a normoxic chamber with a normal oxygen environment (21% inspired O\(_2\) fraction). Eight groups of chronic hypoxic C57Bl/6J mice (21 days of 10% O\(_2\); n=4) were investigated for acute hemodynamic effects of Bay41-2272 and Bay58-2667. After a stabilization period...
of 15 minutes, each group of mice received a different dose of Bay41-2272 and Bay58-2667 (0, 1, 3, or 10 mg/kg) by gavage, and hemodynamics were recorded for 180 minutes.

In a separate set of experiments, telemetric sensors were implanted in wild-type mice, and the effect of a single oral dose of Bay41-2272 or Bay58-2667 (10 mg/kg body wt each) on SAP and heart rate was monitored over a time range of 30 hours.

For assessment of long-term effects of sGC activation, 3 subgroups of animals were treated once per day with either Bay41-2272 (10 mg/kg body wt; n = 10), Bay58-2667 (10 mg/kg body wt; n = 10), or vehicle (methylcellulose 3% at 10 μL/g body wt; n = 10) from day 21 to 35. Hemodynamics were measured as described above.

**Plasma Level of Bay41-2272 and Bay58-2667**

Samples were subjected to high-performance liquid chromatography performed on a 2300 HTLC system (Coesive Technologies) as described. Briefly, the mobile phase consisted of 10 mmol/L ammonium acetate (pH 3.0) and acetonitrile. A linear gradient from 20% to 85% acetonitrile (vol/vol) within 1 minute was applied.

**Tandem mass spectrometry** was performed on an API 3000 triple-quadruple mass spectrometer (PE Sciex) connected to the 2300HTLC system through a Turbospray interface. The lower limit for quantification of Bay41-2272 and Bay58-2667 was 0.5 μg/L.

**Figure 2.** Telemetric measurement of SAP (A) and heart rate (B) in normoxic conscious mice receiving Bay41-2272 or Bay58-2667 and plasma concentrations of Bay41-2272 and Bay58-2667 (C) in chronic hypoxic mice. Continuous 30-hour profile of the deviation of mean blood pressure (A) and heart rate (B) in normoxic mice receiving a single oral application of Bay41-2272 (10 mg/kg body wt) or Bay58-2667 (10 mg/kg body wt) is given. For measurement of plasma levels of both compounds, animals were exposed to hypoxia for 35 days. Bay41-2272 or Bay58-2667 was applied daily by gavage from day 21 to 35 in hypoxia-exposed animals (n = 10) each at a dose of 10 mg/kg body wt. Plasma levels of Bay41-2272 and Bay58-2667 are given (C). The samples were collected 6 hours after the last application of the compounds.

**Monocrotaline and Chronic Treatment**

As described previously, hemodynamic and histological changes were examined in rats at 4 (n = 10) and 6 (n = 15) weeks after a single injection of monocrotaline (60 mg/kg SC). Animals that were injected with monocrotaline for 6 weeks received placebo (10 μL/g body wt in 3% methylcellulose). RVSP (in mm Hg) (A) and right to left ventricular ratio (RV/LV+S) (B) are given. *P < 0.05 vs control; †P < 0.05 vs hypoxia 21 days; ‡P < 0.05 vs hypoxia 35 days.

**Tissue Processing**

After SAP and right ventricular pressure were recorded, the animals were exsanguinated, and the lungs and heart were isolated. The right ventricle was dissected from the left ventricle + septum (LV+S), and these dissected samples were dried and weighed to obtain the right to left ventricle plus septum ratio (RV/LV+S).
Histology
After the lungs were flushed with saline solution, they were perfused through the pulmonary artery and through the trachea with a mixture of formaldehyde (2%) and picric acid (15%) in 0.1 mol/L phosphate buffer with a constant pressure of 22 and 11 cm H₂O, respectively. The lung and the heart were removed en block. The lung lobes were embedded in paraffin blocks, and sections of 3 μm were cut. The degree of muscularization of small peripheral pulmonary arteries was assessed by double staining the 3-μm sections with an anti-α-smooth muscle actin antibody (dilution 1:900, clone 1A4, Sigma, Saint Louis, Mo) and anti-human von Willebrand factor antibody (dilution 1:900, Dako, Hamburg, Germany), as previously described.23 Sections were counterstained with methyl green and examined by light microscopy with the use of a computerized morphometric system (Qwin, Leica). At ×40 magnification, 80 to 100 intra-acinar vessels accompanying either alveolar ducts or alveoli were analyzed by an observer blinded to treatment in each animal. As described, each vessel was categorized as nonmuscularized, partially muscularized, or fully muscularized.24 The percentage of pulmonary vessels in each muscularization category was determined by dividing the number of vessels in that category by the total number counted in the same experimental group.

Figure 5. Effect of Bay41-2272 and Bay58-2667 on RVSP (A) and right heart hypertrophy (B) in NOS3−/− mice. Animals were exposed to hypoxia for 21 or 35 days or remained in normoxia throughout. The sGC activators Bay41-2272 or Bay58-2667 were applied daily by gavage from day 21 to 35 in hypoxia-exposed animals (n=10) each at a dose of 10 mg/kg body wt. Control animals received placebo (10 μL/g body wt in 3% methylcellulose). RVSP (in mm Hg) (A) and right to left ventricular ratio (RV/LV+S) (B) are given. *P<0.05 vs control; †P<0.05 vs hypoxia 21 days.

Effects of 2-Week Daily Oral Administration of Bay41–2272 and Bay58–2667 on SAP, Hematocrit, and Body Weight in Mice With Hypoxia-Induced Pulmonary Hypertension

<table>
<thead>
<tr>
<th>Variable/Intervention</th>
<th>SAP, mm Hg</th>
<th>Hematocrit, %</th>
<th>Body Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type normoxia</td>
<td>77.3±5.2</td>
<td>43.6±0.4</td>
<td>26.5±0.6</td>
</tr>
<tr>
<td>Wild-type hypoxia 3 wk</td>
<td>70.8±4.3</td>
<td>63.3±2.1</td>
<td>22.8±0.5</td>
</tr>
<tr>
<td>Wild-type hypoxia 5 wk</td>
<td>64.5±6.6</td>
<td>57.8±2.4</td>
<td>22.8±0.6</td>
</tr>
<tr>
<td>Wild-type hypoxia/Bay41–2272</td>
<td>62.5±2.8</td>
<td>54.7±1.1</td>
<td>23.9±0.6</td>
</tr>
<tr>
<td>Wild-type hypoxia/Bay58–2667</td>
<td>64.6±5.7</td>
<td>58.9±1.3</td>
<td>21.6±0.6</td>
</tr>
<tr>
<td>NOS3−/− normoxia</td>
<td>107.6±11.0</td>
<td>36.5±2.0</td>
<td>23.7±1.7</td>
</tr>
<tr>
<td>NOS3−/− hypoxia 3 wk</td>
<td>83.2±2.9</td>
<td>62.0±2.1</td>
<td>20.6±0.9</td>
</tr>
<tr>
<td>NOS3−/− hypoxia 5 wk</td>
<td>83.5±8.4</td>
<td>64.6±2.1</td>
<td>24.3±0.6</td>
</tr>
<tr>
<td>NOS3−/− hypoxia/Bay41–2272</td>
<td>80.6±2.6</td>
<td>65.2±2.1</td>
<td>23.9±0.7</td>
</tr>
<tr>
<td>NOS3−/− hypoxia/Bay58–2667</td>
<td>75.0±3.9</td>
<td>65.9±2.3</td>
<td>22.6±0.5</td>
</tr>
</tbody>
</table>

Data are mean±SEM; n=7 to 10.

Western Blot Analysis
Protein concentrations were determined according to Lowry et al.25 Tissue homogenates (15 μg protein per lane) were separated by SDS-PAGE (8%), transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech), and blocked with 3% nonfat dry milk in TBS (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, 0.1% Tween-20). As described previously, immunodetection of sGCα1 and sGCβ1 subunits was performed with the use of polyclonal rabbit antibodies directed and affinity-purified against synthetic peptide sequences corresponding to human sGCα1 (residues 634 to 647) and sGCβ1 (residues 593 to 614), respectively.26,27 Anti-sGCα1 was diluted 1:3000 and anti-sGCβ1 1:2000 in the aforementioned blocking solution. Immune complexes were visualized with an ECL (enhanced chemiluminescence) immunodetection kit (Amersham Pharmacia Biotech). The 80-kDa band for sGCα1 and the 70-kDa band for sGCβ1 were quantitated using ImageJ (National Institutes of Health). The sGC activators Bay41–2272 and Bay58–2667 were applied daily by gavage from day 21 to 35 in hypoxia-exposed animals (n=10) each at a dose of 10 mg/kg body wt. Control animals received placebo (10 μL/g body wt in 3% methylcellulose). Proportions of nonmuscularized (N), partially muscularized (P), or fully muscularized (M) pulmonary arteries was assessed by double staining the 3-μm sections with an anti-α-smooth muscle actin antibody, followed by incubation with a polyclonal rabbit antibody directed against human sGCα1 (dilution 1:900, Dako, Hamburg, Germany), as previously described.23 Sections were counterstained with methyl green and examined by light microscopy with the use of a computerized morphometric system (Qwin, Leica). At ×40 magnification, 80 to 100 intra-acinar vessels accompanying either alveolar ducts or alveoli were analyzed by an observer blinded to treatment in each animal. As described, each vessel was categorized as nonmuscularized, partially muscularized, or fully muscularized.24 The percentage of pulmonary vessels in each muscularization category was determined by dividing the number of vessels in that category by the total number counted in the same experimental group.

Figure 6. Effects of Bay41–2272 and Bay58–2667 on the degree of muscularization of pulmonary arteries in wild-type mice. Animals were exposed to hypoxia for 21 or 35 days or remained in normoxia throughout. The sGC activators Bay41–2272 or Bay58–2667 were applied daily by gavage from day 21 to 35 in hypoxia-exposed animals (n=10) each at a dose of 10 mg/kg body wt. Control animals received placebo (10 μL/g body wt in 3% methylcellulose). Proportions of nonmuscularized (N), partially muscularized (P), or fully muscularized (M) pulmonary arteries, as percentage of total pulmonary artery cross section (sized 20 to 70 μm), are given. A total of 80 to 100 intra-acinar vessels were analyzed in each lung. *P<0.05 vs control; †P<0.05 vs hypoxia 21 days; ‡P<0.05 vs hypoxia 35 days.
band for sGCβ1 were scanned and quantified with a Kodak Image Station IS 440F and normalized to the housekeeping gene β-actin.

Data Analysis
All data are given as mean±SEM. Differences between groups were assessed by ANOVA and Student-Newman-Keuls post hoc test for multiple comparisons, with a probability value <0.05 regarded to be significant.

Results
Effects of Bay41-2272 and Bay58-2667 on Acute Hypoxic Pulmonary Vasoconstriction in Isolated Mouse Lungs
Both Bay41-2272 and Bay58-2667 decreased acute hypoxic pulmonary vasoconstriction in a dose-dependent manner. The maximum inhibitory effect on hypoxic pulmonary vasoconstriction was similar for the 2 agents, but the concentration required to induce a 50% decrease in pulmonary artery pressure for Bay41-2272 was ≈10 times higher than for Bay58-2667 (Figure 1).

Immediate Vasodilatory Effects of Bay41-2272 and Bay58-2667 in Mice With Hypoxia-Induced Chronic Pulmonary Hypertension
Both compounds reduced right ventricular systolic pressure in a dose-dependent manner from 1 to 10 mg/kg body wt (Figure 2). Pulmonary vasodilatation was accompanied by a decrease in SAP. Telemetric measurement showed that 1 oral administration of either Bay41-2272 or Bay58-2667 (dose 10 mg/kg) reduced SAP by ≈20% over a time range of 10 to 20 hours (Figure 3A). Heart rate ranged from ≈600 bpm and increased to ≈700 bpm in response to the compounds (Figure 3B). This value normalized ≈5 hours after oral application of Bay41-2272 or Bay58-2667. Plasma samples were collected 6 hours after the last application of the compounds, and the levels of Bay41-2272 and Bay58-2667 were measured at 10 and 25 nmol/L, respectively (Figure 3C).

Chronic Effects of Bay41-2272 and Bay58-2667 on Hemodynamics and Right Heart Hypertrophy in Mice With Hypoxia-Induced Pulmonary Hypertension
The hypoxic wild-type mice developed pulmonary hypertension within 21 days, which was sustained until day 35.

Figure 7. sGC α1 and β1 subunit expression in hypoxia (hox)-induced pulmonary hypertension in wild-type (A) and NOS3−/− (B) mice. Western blot analysis was used to assess expression of sGC α1 and β1 subunits in lungs from controls, hypoxia-challenged animals, and animals treated with Bay41-2272 or Bay58-2667. Densitometric analysis of sGC subunit expression is given and normalized to the housekeeping gene β-actin (C, D). Immunoblots are representative of n=3 to 5 blots for each group.
Consequently, right ventricular systolic pressure (RVSP) was increased significantly compared with the control group (Figure 4A). This increase was accompanied by an increase in the ratio of right ventricle to left ventricle plus septum weight [RV/(LV + S)] (Figure 4B). The ratio increased from 0.24 ± 0.02 (controls) to 0.38 ± 0.02 (21 days of hypoxia) and 0.42 ± 0.03 (35 days of hypoxia), respectively (both P < 0.05 versus controls). Bay41-2272 and Bay58-2667, applied by gavage from day 21 to 35, significantly reduced hypoxia-induced chronic pulmonary hypertension in wild-type mice. Accordingly, Bay41-2272 and Bay58-2667 caused a decrease of the RV/(LV+S) ratio to 0.32 ± 0.02 and 0.31 ± 0.02, respectively. Mean SAP did not change in any of the treatment groups (Table). Likewise, NOS3−/− mice developed pulmonary hypertension, with RVSP values increasing from 23.7 ± 0.8 (controls) to 35.5 ± 3.0 (21 days of hypoxia) and 34.9 ± 1.2 (35 days of hypoxia) (Figure 5A) and RV/(LV+S) values increasing from 0.24 ± 0.01 (controls) to 0.34 ± 0.02 (21 days of hypoxia) and 0.41 ± 0.08 (35 days of hypoxia) (Figure 5B). Bay58-2667, but not Bay41-2272, caused a moderate but significant reduction of RVSP in NOS3−/− mice, whereas both compounds failed to reduce RV/(LV+S) values (Bay41-2272, 0.36 ± 0.02; Bay58-2667, 0.39 ± 0.06). Mean SAP did not change in any of the treatment groups (Table).

**Chronic Effects of Bay41-2272 and Bay58-2667 on Degree of Muscularization of Pulmonary Arteries in Mice With Hypoxia-Induced Pulmonary Hypertension**

We quantitatively assessed the degree of muscularization of pulmonary arteries with a diameter from 20 to 70 μm. In wild-type mice, the majority of vessels from 20 to 70 μm are usually nonmuscularized and partially muscularized (Figure 6). In the hypoxia-exposed animals, both at day 21 and 35, a dramatic decrease in nonmuscularized pulmonary arteries occurred with a concomitant increase in fully and partially muscularized pulmonary arteries. Treatment with Bay41-2272 and Bay58-2667 resulted in a significant increase of nonmuscularized arteries compared with both hypoxia groups. In addition, Bay41-2272 decreased the percentage of partially muscularized pulmonary arteries.

**Expression of α and β Subunits of sGC in Mice With Hypoxia-Induced Pulmonary Hypertension: Effects of Bay41-2272 and Bay58-2667**

The protein levels of both subunits of sGC did not change significantly in response to hypoxia (Figure 7). In contrast, in NOS3−/− mice, the α1 subunit of sGC was downregulated at day 35 and the β1 subunit decreased at day 35. Significant changes in either sGCα1 or sGCβ1 subunit appeared in none of the treatment groups.

**Chronic Effects of Bay41-2272 and Bay58-2667 on Hemodynamics and Right Heart Hypertrophy in Rats With Monocrotaline-Induced Pulmonary Hypertension**

In rats injected with monocrotaline for 28 days, severe pulmonary hypertension developed with marked increase in RVSP (from 25.1 ± 1.4 to 67.7 ± 3.1 mm Hg; Figure 8A), in the ratio of right ventricular weight to left ventricle plus septum (RV/LV+S) (from 0.30 ± 0.01 to 0.63 ± 0.01; Figure 8B), and in the percentage of pulmonary artery muscularization (Figure 9A, 9B). In rats treated with vehicle, further progression of pulmonary hypertension until day 42 was noted (RVSP = 78.5 ± 6.2 mm Hg; RV/LV+S = 0.81 ± 0.05; Figures 8 and 9). No significant changes in mean SAP were observed (control = 117 ± 9 mm Hg; monocrotaline for 4 weeks = 103 ± 9 mm Hg; monocrotaline for 6 weeks = 109 ± 10 mm Hg). Ninety percent (9/10) and 60% (9/15) of animals survived the 28- and 42-day monocrotaline treatment. Long-term treatment with Bay41-2272 or Bay58-2667 significantly decreased RVSP to 55.5 ± 1.7 and 53.9 ± 2.9 mm Hg, respectively (P < 0.05 versus monocrotaline both at day 42 and at day 28). In addition, both compounds decreased RV/LV+S values to 0.47 ± 0.01 and 0.50 ± 0.03, respectively (P < 0.05 versus monocrotaline both at day 42 and at day 28). SAP was unchanged (Bay41-2272, 91 ± 4 mm Hg; Bay58-2667, 102 ± 6 mm Hg). In the animals treated with Bay41-2272 or Bay58-2667, survival was 80% (8/10) and 70% (7/10), respectively.

**Chronic Effects of Bay41-2272 and Bay58-2667 on Degree of Muscularization of Pulmonary Arteries in Rats With Monocrotaline-Induced Pulmonary Hypertension**

We quantitatively assessed the degree of muscularization of pulmonary arteries with a diameter from 10 to 50 μm. In the
monocrotaline-injected animals, both at day 28 and 42, a significant decrease in nonmuscularized pulmonary arteries occurred (Figure 9A) with a concomitant increase in fully muscularized pulmonary arteries. Treatment with Bay41-2272 or Bay58-2667 at 10 mg/kg per day resulted in a significant reduction of fully muscularized arteries and increased the percentage of nonmuscularized pulmonary arteries (both parameters $P<0.05$ versus monocrotaline both at day 42 and at day 28) (Figure 9B).

**Discussion**

In this study we demonstrated that both the sGC stimulator Bay41-2272 and the sGC activator Bay58-2667 reverse pulmonary hypertension in chronically hypoxic mice and monocrotaline-injected rats. Notably, treatment with these agents was commenced only after full establishment of pulmonary hypertension, right heart hypertrophy, and structural changes in the lung vasculature. The compound Bay41-2272 is a novel NO-independent stimulator of sGC with characteristics similar to YC-1 but with higher potency of $\approx 2$ to 3 orders of magnitude and no phosphodiesterase-5 inhibitory activity. Bay41-2272 also acts synergistically with NO, which was shown experimentally in NO-dependent penile erection and experimental acute pulmonary hypertension. In both systems, the NO/sGC/cGMP system plays an important role in maintaining physiological function. In contrast, the compound Bay58-2667 does not synergize with NO but stimulates the heme-oxidized or heme-depleted purified enzyme (Figure 10). Both compounds Bay41-2272 and Bay58-2667 are orally bioavailable, and both proved to have a long-lasting effect over 10 and 12 hours, respectively. Similarly, we show in our study in mice that both compounds Bay41-2272 and Bay58-2667 reduce SAP for $\approx 20$ hours. On the basis of these findings, therapy was performed by once-daily application to achieve optimal efficacy. Detailed pharmacokinetic studies have been performed for Bay41-2272.
conditions has been addressed by several groups. Although the aortic GC content was not altered in NOS3 knockout animals or NOS inhibitor–treated rats, hypertension and aging appear to result in downregulation of GC expression. In experimental models of hypoxia-induced pulmonary hypertension, upregulation of sGC expression has been reported in rats and mice. With the use of immunostaining and Western blotting, a >2-fold increase of sGC protein α1 subunit was noted in smooth muscle cells of the pulmonary arteries in hypoxic rat lungs. The same group demonstrated in mice that both subunits of sGC, the α1 and β1 subunits, were increased under conditions of hypoxia-induced pulmonary hypertension. Interestingly, similar results were observed in NOS2 knockout animals but not in NOS3 knockout animals, suggesting NOS3 as a major regulator of sGC activity and protein expression in the lung vasculature. In this study both the α1 and β1 subunits were not changed in wild-type mice in response to hypoxia, and the expression was not altered by the 2 sGC activators. In contrast, a downregulation was noted in NOS3 mice, which is well in agreement with the aforementioned previous report.

Against this background, sGC is an attractive target for the treatment of hypoxia-induced pulmonary vascular diseases. In contrast to previous investigations, which investigated the influence of an endothelin antagonist, prostaglandin E1, or phosphodiesterase-5 inhibitors together with the hypoxic challenge, we started therapeutic interventions when pulmonary hypertension was already fully established, from week 3 to 5 in mice and from week 4 to 6 in rats. Under these conditions, both Bay41-2272 and Bay58-2667 significantly reversed the degree of pulmonary hypertension evolving in response to hypoxia and monocrotaline. This was true for systolic pulmonary artery pressure and right heart hypertrophy in these mice. These findings suggest that the antiremodeling effects of Bay41-2272 and Bay58-2667 were both dependent on ongoing NOS3-dependent NO generation.

The NOS3 mice developed pulmonary hypertension with hemodynamics and morphological changes similar to those in wild-type mice, which is in contrast to 1 previous report but is in agreement with 2 other publications. Interestingly, both compounds failed to reverse RVSP and right heart hypertrophy in these mice. These findings suggest that the antiremodeling effects of Bay41-2272 and Bay58-2667 were both dependent on ongoing NOS3-dependent NO generation.

The antiremodeling potency of Bay58-2667 in hypoxic mice is particularly interesting because this compound activates mainly the oxidized or the heme-free form of sGC, which does not occur under physiological conditions. However, it has recently been shown that in lungs from mice kept under hypoxic conditions, levels of reactive oxygen species may even increase, which in turn might oxidize the heme group of sGC. Enhanced levels of oxygen radicals have also been found under conditions of atherosclerosis, diabetes, hypercholesterinemia, or hypertension. However, future studies must prove the incidence of heme-free or oxidized forms of sGC in vascular abnormalities such as chronic pulmonary hypertension.

In conclusion, the compounds Bay41-2272 and Bay58-2667 caused dose-dependent pulmonary vasodilation in hyp-
oxia-induced pulmonary hypertension in isolated mouse lungs. When these agents are used for chronic treatment by daily gavage, reversal of the hypoxia-elicited pulmonary hypertension was demonstrated, which was true for hemodynamics, structural changes of the lung vasculature, and right heart hypertrophy. Notably, the efficacy of both agents was dependent on intact NOS function. We conclude that activation of sGC may offer a new therapeutic option for antiremodeling therapy in severe pulmonary hypertension.

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Disclosures

Drs Stasch and Gnoth report that they are employed by Pharma Research Center, Bayer HealthCare. The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

Blood vessel remodeling in the context of chronic systemic and pulmonary disorders (eg, systemic and pulmonary hypertension, chronic obstructive pulmonary disease, interstitial lung disease, left heart failure, diabetes, and arteriosclerosis) shares many similarities such as medial wall thickening, neointimal formation, and endothelial dysfunction. The nitric oxide (NO)–soluble guanylate cyclase (sGC) pathway plays a central role in maintaining physiological organ function. Alterations of this pathway have been attributed to be centrally involved in the course of these diseases and are subject to the development of new therapeutic agents. Among the most recent approaches, approval of the phosphodiesterase-5 inhibitor sildenafil for the treatment of pulmonary arterial hypertension represents the most intriguing therapeutic option. In the present study we address another important molecular key player of the NO/cGMP axis by proving the therapeutic efficacy of the sGC stimulator Bay41-2272 and activator Bay58-2667 in 2 well-established models of chronic pulmonary hypertension (hypoxia and monocrotaline-induced pulmonary hypertension). Both compounds not only improved pulmonary hemodynamics symptomatically (as previously shown for many other substances) but also reversed vascular remodeling. Notably, treatment with these agents was commenced after full establishment of pulmonary hypertension, right heart hypertrophy, and structural changes in the lung vasculature. Targeting sGC is of considerable interest because stimulators and activators of this enzyme represent a new class of drugs complementary to currently established therapies for chronic vascular disorders (eg, phosphodiesterase inhibitors, ACE inhibitors, endothelin receptor antagonists). Clinical trials are warranted to address the safety and efficacy of these substances.
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