ABSTRACT

Phosphodiesterase type 4 (PDE4) is involved in the hydrolysis of cAMP in pulmonary vascular smooth muscle (PA-SMC) and immune inflammatory cells. Given that intracellular cAMP accumulation inhibits contraction and growth of PA-SMCs as well as inflammatory cell functions, we investigated the effects of the PDE4 inhibitor 3-cyclopentylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide (roflumilast), on pulmonary hypertension (PH) in rats. Treatment with roflumilast (0.5 or 1.5 mg·kg⁻¹·day⁻¹) from day 1 to day 21 after monocrotaline (MCT) injection (60 mg·kg⁻¹ s.c.) attenuated PH development: pulmonary artery pressure, right ventricular hypertrophy, and muscularization of distal vessels on day 21 were decreased compared to control MCT-treated rats. Roflumilast (1.5 mg·kg⁻¹·day⁻¹) also reduced the increases in interleukin-6 and monocyte chemotactic protein-1 mRNAs observed in lung tissue on day 21 without affecting the rise in interleukin-1β mRNA on days 1 and 21. Roflumilast (1.5 mg·kg⁻¹·day⁻¹) from day 21 to day 42 after MCT reversed established PH, almost normalizing pulmonary artery pressure and structure, and suppressing proliferating cell nuclear antigen-positive cells in pulmonary vascular walls. Treatment with roflumilast similarly attenuated PH development due to chronic hypoxia. Treatment of human PA-SMCs with roflumilast N-oxide, the active metabolite of roflumilast, at concentrations up to 10⁻⁶ M, potentiated PA-SMC growth inhibition induced by prostacyclin (10⁻⁵ M) or interleukin-1β (10 ng·ml⁻¹) but was inactive on its own. In conclusion, the PDE4 inhibitor roflumilast significantly attenuates pulmonary vascular remodeling and hypertension induced by chronic hypoxia or MCT and reverses established PH after MCT administration.

Pulmonary hypertension (PH) is characterized by an increase in pulmonary vascular resistance that impedes ejection of blood by the right ventricle and subsequently leads to right ventricular failure. The most common form of PH occurs in patients with chronic hypoxemic lung disease such as chronic obstructive pulmonary disease (COPD). Although PH is usually mild to moderate in this condition and stabilized with long-term oxygen therapy, it remains the strongest prognostic factor, independent from the severity of air flow limitation or hypoxemia (Burrows et al., 1972). Among the many other causes of PH, idiopathic pulmonary hypertension and PH associated with inflammatory conditions such as connective tissue diseases are severe and often fatal diseases, although new treatments help to prolong survival and quality of life.

Persistent vasoconstriction and structural remodeling of pulmonary vessels with proliferation of vascular smooth muscle cells (SMCs) are cardinal features of PH. An imbalance between vasoactive factors such as endothelin and serotonin, which exhibit constrictor and mitogenic properties on pulmonary vascular smooth muscle, and endogenous vasodilator substances such as prostacyclin, which not only relax pulmonary vascular smooth muscle but also exert an-

Effects of Roflumilast, a Phosphodiesterase-4 Inhibitor, on Hypoxia- and Monocrotaline-Induced Pulmonary Hypertension in Rats

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timitogenic effects, has been shown to play a major role in the pathogenesis of PH (Christman et al., 1992; Giaid et al., 1993; Giaid and Saleh, 1995). Several lines of evidence also indicate that inflammatory events are involved in the pathogenesis of PH (Tuder and Voelkel, 1998). Lung proinflammatory cytokines and chemokines are produced in excess in patients with severe PH (Tuder and Voelkel, 1998), as well as in the experimental models of monocrotaline (MCT) or hypoxia-induced PH, suggesting that they may play a role in the proliferation of vascular SMCs (Guignabert et al., 2005).

Among the various compounds that have been shown to exert preventive or therapeutic effects in experimental PH are drugs that elevate intracellular cAMP or cGMP levels (Schermuly et al., 2004). Intracellular cAMP hydrolysis is regulated by the enzymes of the phosphodiesterase (PDE) superfamily. Phosphodiesterase type 4 (PDE4) is involved in the metabolism of cAMP in pulmonary vascular and bronchial SMCs, as well as in immune inflammatory cells (Rabe et al., 1994; Torphy, 1998). The investigational PDE4 inhibitor 3-cyclopropylmethoxy-4-difluoromethoxy-N-[3,5-di-chloropyrid-4-yl]-benzamide (roflumilast) was recently shown to be associated with an improvement of lung function in COPD patients, an effect related in part to a reduction of lung inflammation (Rabe et al., 1994). In vitro, in vivo, and clinical investigations substantiate the strong anti-inflammatory potential of roflumilast (Rabe et al., 2005; Boswell-Smith and Page, 2006). In view of previous data demonstrating that endogenous prostacyclin, whose main effects are mediated by intracellular cAMP, attenuates the development of PH and the well-established concept that prostacyclin analogues exert antiproliferative effects on PA-SMCs, we speculated that specific PDE4 inhibitors may have beneficial effects in PH by protecting against pulmonary vascular remodeling and inflammation.

Therefore, the purpose of this study was to investigate whether roflumilast attenuated the development of PH and reversed the established PH. To address this question, we used two rat models of PH, the hypoxic model that, although not including lung parenchyma destruction, is reminiscent of the PH observed in COPD, and the MCT model characterized by severe PH with no spontaneous recovery. We further examined the effect of roflumilast on the increased cytokine production we documented previously in lung tissue of rats with MCT-induced PH. In addition, we investigated the antiproliferative effects of roflumilast N-oxide, the active metabolite of roflumilast (Bethke et al., 2006), in cultured human PA-SMCs.

Materials and Methods

Materials

Pathogen-free adult male Wistar rats were acquired from Charles River Laboratories (Les Oncins, France). Monocrotaline, polyethylene glycol 400, indomethacin, pepstatin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, benzamidine, and cAMP were purchased from Sigma-Aldrich Chimie S.a.r.l. (Lyon, France). TRIzol, SuperScript II reverse transcriptase system, RNase-Out, fetal bovine serum (FBS), and Dulbecco’s modified Eagle’s medium (DMEM) were from Invitrogen (Paisley, UK). Random hexamer primers, deoxynucleotide triphosphate mix, [methyl-3H]thymidine, [3H]cAMP, and QAE Sephadex A-25 were acquired from Amersham Pharmacia Biotech (Chalfont St. Giles, UK). Target retrieval solution, anti-proliferating cell nuclear antigen (PCNA) mouse monoclonal antibody, and the Universal LSAB+ (horseradish peroxidase) detection kit were from Dako Denmark AS (Glostrup, Denmark). Prostacyclin was from Cayman Europe (Tallinn, Estonia). Interleukin (IL)-1β was purchased from R&D Systems (Minneapolis, MN). RNaseasy Mini kit was obtained from Qiagen (Courtaboeuf, France). Syber Green Mix was acquired from Applied Biosystems (Foster City, CA). The enzyme-linked immunosorbent assay kit for determination of 6-keto PGF1α was purchased from Biotrend (Cologne, Germany). Biacinchoninic acid assay was from Pierce Chemical (Rockford, IL). Roflumilast and roflumilast N-oxide were synthesized at the chemical facilities of Nycomed Research & Development (formerly Altana Pharma AG) essentially as described in Amschler et al. (1995).

Animal Models and Experimental Design

Pulmonary Hypertension-Induced by CH or MCT. All experiments were performed using adult male Wistar rats (200–250 g) according to institutional guidelines that comply with national and international regulations. Pulmonary hypertension was induced in response to CH by exposing rats to 10% O2 in a ventilated chamber (500-L volume; Flufrance, Cachan, France) for 15 days. To establish the hypoxic environment, the chamber was flushed with a mixture of room air and nitrogen, and the gas was recirculated. The chamber environment was monitored using an oxygen analyzer (OxiQuant M; Envitec, Wismar, Germany). Carbon dioxide was removed by soda lime granules, and excess humidity was prevented by cooling the recirculation circuit. The chamber temperature was maintained at 22–24°C. The chamber was opened every other day for 1 h to clean the cages and replenish food and water supplies. Normoxic control rats were kept in the same room with the same light-dark cycle. For studies in MCT-induced PH, rats exposed to normoxia received a single injection of MCT (60 mg·kg−1·s.c.) and were studied on day 21 or 42.

Treatment of Animals with Roflumilast. To assess the potential preventive effect of PDE4 inhibition in hypoxia- and MCT-induced PH, we initially assigned rats at random to eight groups. Three groups were treated with MCT, and three groups were exposed to CH. Among these MCT- and hypoxia-exposed groups, two groups were treated with roflumilast (0.5 and 1.5 mg·kg−1·day−1, respectively) and one group was treated with vehicle. Two additional groups maintained under normoxic condition were treated with roflumilast (1.5 mg·kg−1·day−1) and vehicle, respectively. All treatments were given once a day by gavage as used previously (Kumar et al., 2003).

Finally, to assess the potential effect of PDE4 inhibition on established PH, rats given MCT (60 mg·kg−1·s.c.) were left untreated for 21 days and then randomly divided into two groups: one group treated was with roflumilast (1.5 mg·kg−1·day−1) and the other group was treated with vehicle from day 21 to day 42. The effect of roflumilast (1.5 mg·kg−1·day−1) on survival was evaluated from the date of MCT injection to day 42.

Assessment of Pulmonary Hypertension

Rats were anesthetized with sodium pentobarbital (60 mg·kg−1·i.p.). A polyvinyl catheter was introduced into the right jugular vein and pushed through the right ventricle into the pulmonary artery. A polyethylene catheter was inserted into the right carotid artery. After measurement of pulmonary (PAP) and systemic (SAP) arterial pressures, the thorax was opened, and the left lung was immediately removed and frozen in liquid nitrogen. The heart was dissected and weighed for calculation of the right ventricular hypertrophy index (ratio of right ventricular free wall weight over sum of septum plus left ventricular free wall weight, RV/LV + S). The right lung was fixed in the distended state with formalin buffer. After routine processing and paraffin embedding, multiple sections from each lobe were stained with hematoxylin-eosin and used for morphometric analysis.
were stained with hematoxylin and eosin. In each rat, 60 intracinar arteries were analyzed and categorized as muscular (fully or partially) or nonmuscular to assess the degree of muscularization. In addition, fully muscularized intracinar arteries were evaluated for measurements of medial wall thickness calculated and expressed as follows: index (percentage) = (external diameter – internal diameter)/external diameter × 100.

Real-Time Quantitative Reverse-Transcription-PCR for Measurement of the Effect of PDE4 Inhibition on IL-1β, MCP-1, and IL-6 mRNA Expression in MCT Rat Lungs

IL-1β, monocyte chemotactic protein-1 (MCP-1), and IL-6 mRNA expression were measured in rat lungs on days 1 and 21 after monocrotaline injection in animals treated with roflumilast (1.5 mg · kg⁻¹ · day⁻¹) or vehicle. RNA was extracted using TRIzol reagent (Invitrogen). RNA concentration and quality were determined by electrophoresis on agarose gel and spectrophotometry. Then, reverse transcription was performed using random hexamer primers and reverse transcriptase (Invitrogen). Primers for PCR were designed with Primer Express software (Applied Biosystems). To avoid inappropriate amplification of residual genomic DNA, intron-spanning primers were selected and internal control 18S ribosomal RNA primers were provided. First-strand cDNA synthesis was carried out using the SuperScript II reverse transcriptase system (Invitrogen). One microgram of total RNA, 2 μl of deoxynucleotide triphosphate mix (10 mM), and 100 ng of random primers in a total volume of 12 μl were incubated for 5 min at 65°C and chilled on ice. Four microliters of first-strand buffer, 2 μl of dithiothreitol (0.1 M), and 40 U of ribonuclease inhibitor (Invitrogen) were added to the samples and heated at 42°C for 2 min. Finally, after adding 1 μl of SuperScript reverse transcriptase II (200 units/μl), the reaction was incubated for 10 min at 25°C, 50 min at 42°C, and 15 min at 70°C.

For each sample, the amplification reaction was performed in duplicate using Syber Green Mix and specific primers. Signal detection and analysis of results were performed using ABI-Prism 7000 sequence detection software (Applied Biosystems). The relative expression level of the gene of interest was computed with respect to the mRNA expression level of the internal standard, r18S, using the following formula: relative mRNA concentration = 2(NCt gene of interest – Ct r18S).

In Situ Proliferation of Rat PA-SMCs

To determine the mechanism by which roflumilast (1.5 mg · kg⁻¹ · day⁻¹) diminished PH induced by monocrotaline, PCNA labeling was evaluated in rat lung sections. Tissue sections were deparaffinized in xylene and then treated with graded series of alcohol washes, rehydrated in PBS, pH 7.5, and incubated with target retrieval solution in a water bath at 90°C for 20 min. Endogenous peroxidase activity was blocked with H₂O₂ in PBS (3%, v/v) for 5 min. Slides were then washed in PBS and incubated for 30 min with anti-PCNA mouse monoclonal antibody (PC-10). Antibodies were washed off, and the slides were processed with the Universal LSAB+ (horseradish peroxidase) detection kit. Brown color was generated with a diaminobenzidine substrate, and nuclei were counterstained with hematoxylin.

Effects of Roflumilast N-Oxide on PA-SMC Proliferation Assessed by [³H]Thymidine Incorporation

Human PA-SMCs were cultured from explants of pulmonary arteries as described previously (Eddahibi et al., 2001) or purchased from Promocell (Heidelberg, Germany). PA-SMCs were plated in 24-well format, cultured in DMEM with 10% FBS, and growth-arrested in serum-free medium for 48 h. PA-SMCs were then preincubated with roflumilast N-oxide (10 pm–1 μM), IL-1β (10 ng · ml⁻¹), indomethacin (1 μM), or prostacyclin (1 μM) or with vehicle (DMSO) for 15 min followed by stimulation with 5% FBS. [methyl-³H]Thymidine (1 μCi/well) was added over the last 4 h of a 24-h incubation period. Cells were washed twice with PBS, exposed to ice-cold 10% (w/v) trichloroacetic acid, and dissolved in 0.1 N NaOH (0.5 ml/well). Incorporated radioactivity was counted and expressed as cpm per well. Roflumilast N-oxide and indomethacin were dissolved from stock solutions in DMSO, and final DMSO concentrations were 0.2% in all conditions.

Determination of 6-Keto PGF₁α in PA-SMC Culture Media

Growth-arrested human PA-SMCs were preincubated with indomethacin (10 μM) or vehicle (DMSO) and cultured with IL-1β (10 ng · ml⁻¹) or buffer control for 18 h. The stable prostacyclin metabolite 6-keto PGF₁α was measured in culture media using commercially available enzyme-linked immunosorbent assay and expressed as picograms per milliliter.

PDE4 Activity Measurements

Growth-arrested human PA-SMCs were preincubated with indomethacin (10 μM) or vehicle (DMSO) and then cultured with IL-1β (10 ng · ml⁻¹) or buffer control in DMEM. After 6 h, the culture dishes were washed twice in phosphate-buffered saline (4°C), and the cells were scraped in 1 ml of homogenization buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 10 mM HEPES, 1 mM EDTA, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 5 μM peptatin A, 10 μM leupeptin, 50 μM phenylmethylsulfonyl fluoride, 10 μM soybean trypsin inhibitor, and 2 mM benzamidine, pH 8.2). Cells were disrupted by sonication (3 × 15 s) in a sonifier (Branson Ultrasonics Corporation, Danbury, CT), and lysates were immediately used for PDE activity measurements.

PDE activities were assessed in cellular lysates as described previously (Thompson and Appleman, 1971), with modifications (Bauer and Schwabe, 1980). The assay mixture (200 μl, final volume) contained 30 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 μM CAMP, [³H]AMP (approximately 30,000 cpm/well), 100 μM EGTA, roflumilast N-oxide (1 μM) to inhibit PDE4 or vehicle (0.1% DMSO), and PA-SMC lysate. Incubations were performed for 20 min at 37°C, and reactions were terminated by adding 50 μl of 0.2 M HCl per well. Assays were left on ice for 10 min, and 25 μg of 5'-nucleotidase (Crotalus atrox) was then added. After incubation for 10 min at 37°C, the assay mixtures were loaded on QAE-Sephadex A25 columns (bed volume, 1 ml). Columns were eluted with 2 ml of 30 mM ammonium formate, pH 6.0, and radioactivity in the eluate was counted. PDE4 was calculated as the difference of PDE activities at 0.5 μM CAMP with and without 1 μM roflumilast N-oxide, which completely and selectively inhibited PDE4.

In another approach, lungs from rats were excised at various times after exposure to MCT or vehicle control and rapidly frozen in liquid N₂ before being homogenized in ice-cold homogenization buffer. PDE4 activities in lung homogenates were determined as described above.

PDE activities were calculated as picomoles of CAMP hydrolyzed per minute per milligram of protein. Protein was measured with the bicinchoninic acid assay.

Statistical Analysis

The data are expressed as means ± S.E.M. A nonparametric Mann-Whitney test was used for comparisons between two groups. Comparisons of data at various times after MCT injection or of various treatment groups were performed using a nonparametric Kruskal-Wallis test followed by Dunn’s test when significant. Kaplan-Meier methods were used to obtain survival curves, and a two-sided log-rank test was used to compare strata.

To compare the degree of pulmonary vessel muscularization between groups, we used a nonparametric Mann-Whitney or Kruskal-Wallis test after ordinal classification of the vessels as nonmuscular, partially muscular, or fully muscular. The effect of roflumilast on cytokine expression in the lung at various times after MCT injection was evaluated by two-way analysis of variance, testing for treatment
controls injected with saline instead of MCT (1.5 mg·kg⁻¹ day⁻¹) or vehicle was administered to Wistar rats after exposure to MCT (60 mg·kg⁻¹ s.c., single dose) or chronic hypoxia. Pulmonary artery pressure was measured on day 21 after MCT exposure or after 15 days of hypoxia exposure. Results are shown as means ± S.E.M. from n = 8 (MCT) or n = 9 (chronic hypoxia) rats per group. *, p < 0.05 and **, p < 0.01 compared with MCT- or hypoxia-exposed rats treated with vehicle (Dunn’s test after Kruskal-Wallis test).

Results

Effect of PDE4 Activity Inhibition on Development of PH Induced by MCT or CH. Treatment with roflumilast (1.5 mg·kg⁻¹ day⁻¹) did not affect hemodynamics, RV/LV + S, or muscularization in control rats treated with saline instead of MCT and maintained under normoxic conditions (data not shown). In rats treated with vehicle after MCT administration and studied on day 21, PH developed, with marked increase in PAP, RV/LV + S, and PA muscularization (Figs. 1–3) compared with control rats. Treatment with roflumilast from day 1 to day 21 after MCT attenuated PH development but was without significant effects on body weight, systemic artery pressure, or heart rate (Table 1). Pulmonary artery pressure, RV/LV + S, and muscularization were significantly lower in MCT rats given roflumilast (0.5 or 1.5 mg·kg⁻¹ day⁻¹) than in those given vehicle, the preventive effect of roflumilast on development of PH being more marked with the higher dose. However, the higher dose did not abolish PH, because PAP and muscularization remained greater in MCT rats treated with 1.5 mg·kg⁻¹ day⁻¹ than in controls injected with saline instead of MCT (p < 0.05).

Treatment of rats with roflumilast (1.5 mg·kg⁻¹ day⁻¹) during CH significantly attenuated PH development (Figs. 1–3) but did not affect systemic artery pressure or heart rate (Table 1). Although PAP, right ventricular hypertrophy, and muscularization on day 15 of hypoxia exposure were significantly lower in rats treated with the higher roflumilast dose than in those treated with vehicle, these values remained higher than in normoxic control rats (p < 0.05). Body weight was slightly but significantly lower in hypoxic rats treated with the higher roflumilast dose than in the vehicle-treated group (−7%) (p < 0.05).

Reversal by Roflumilast of MCT-Induced PH. Treatment of MCT rats with roflumilast (1.5 mg·kg⁻¹ day⁻¹) after PH development, from day 21 to day 42, partly reversed PH; on day 42, PAP, RV/LV + S, and PA muscularization were significantly lower in roflumilast-treated animals than in MCT rats studied before (day 21) or after (day 42) treatment with vehicle (Fig. 4, A–C). Moreover, wall thickness of fully muscularized vessels was significantly lower in roflumilast-treated rats than in MCT rats before or after treatment with vehicle (18 ± 2% versus 51 ± 3 and 52 ± 4%, respectively; p < 0.001). In addition, among fully muscularized vessels, the percentage of obliterated vessels was significantly lower in roflumilast-treated rats than in MCT rats before or after treatment with vehicle (30 ± 5% versus 50 ± 1 and 66 ± 3%, respectively; p < 0.01). In MCT rats treated with vehicle, PCNA labeling of lung

![Fig. 1. Prevention by roflumilast of the development of pulmonary hypertension after exposure to MCT or hypoxia. Roflumilast (0.5 or 1.5 mg·kg⁻¹ day⁻¹) or vehicle was administered to Wistar rats after exposure to MCT (60 mg·kg⁻¹ s.c., single dose) or chronic hypoxia. Pulmonary artery pressure was measured on day 21 after MCT exposure or after 15 days of hypoxia exposure. Results are shown as means ± S.E.M. from n = 8 (MCT) or n = 9 (chronic hypoxia) rats per group. *, p < 0.05 and **, p < 0.01 compared with MCT- or hypoxia-exposed rats treated with vehicle (Dunn’s test after Kruskal-Wallis test).

![Fig. 2. Preventive effect of roflumilast on MCT- or hypoxia-induced right ventricular hypertrophy. Roflumilast (0.5 or 1.5 mg·kg⁻¹ day⁻¹) or vehicle was administered to Wistar rats exposed to MCT (60 mg·kg⁻¹ s.c., single dose) or chronic hypoxia. Right ventricular hypertrophy was determined as RV/LV + S ratio on day 21 after MCT or after 15 days of hypoxia. Results are shown as means ± S.E.M. from n = 8 (MCT) or n = 9 (chronic hypoxia) rats per group. *, p < 0.05 and **, p < 0.01 compared with MCT- or hypoxia alone (Dunn’s test after Kruskal-Wallis test).]
Body weight, SAP, and heart rate in rats treated with vehicle or with roflumilast (0.5 or 1.5 mg · kg⁻¹ · day⁻¹) on day 21 after MCT administration or after 15 days of exposure to chronic hypoxia. Exposure to chronic hypoxia was associated with a decrease in body weight in rats treated with roflumilast (1.5 mg · kg⁻¹ · day⁻¹). All values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Body wt</th>
<th>SAP</th>
<th>Heart Rate</th>
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<tr>
<td>MCT</td>
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<tr>
<td>Vehicle (n = 10)</td>
<td>285 ± 5</td>
<td>102 ± 14</td>
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<tr>
<td>ROF 0.5 (n = 8)</td>
<td>285 ± 6</td>
<td>86 ± 5</td>
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<tr>
<td>ROF 1.5 (n = 8)</td>
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<td>102 ± 7</td>
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<tr>
<td>Hypoxia</td>
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<tr>
<td>Vehicle (n = 10)</td>
<td>322 ± 5</td>
<td>123 ± 6</td>
</tr>
<tr>
<td>ROF 0.5 (n = 9)</td>
<td>308 ± 5</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>ROF 1.5 (n = 9)</td>
<td>300 ± 8</td>
<td>110 ± 5</td>
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ROF, roflumilast.
* P < 0.05 compared with values recorded in vehicle-treated rats.

Histologic sections showed proliferation of SMCs in distal PA walls on days 21 and 42, whereas in rats treated with roflumilast (1.5 mg · kg⁻¹ · day⁻¹) from day 21 to day 42, PCNA-positive cells were absent from PA walls (Fig. 5).

Survival was examined in two groups of rats treated from day 21 to day 42 after MCT. On day 42, 17 of 24 rats (71%) treated with roflumilast (1.5 mg · kg⁻¹ · day⁻¹) were alive compared with 21 of 39 rats (54%) treated with vehicle (p < 0.05).

Effects of Roflumilast on Lung Expression of Cytokines during the Development of MCT-Induced PH. Pulmonary expressions of the cytokines IL-1β, IL-6, and MCP-1 shown previously to be involved in the inflammatory response to MCT (Guignabert et al., 2005) were measured in lung tissue on days 1 and 21 after MCT (Fig. 6). There was an early increase in IL-1β mRNA on day 1 and a larger increase on day 21. In contrast, increases in IL-6 and MCP-1 mRNA levels were observed on day 21 but not on day 1. Treatment with roflumilast (1.5 mg · kg⁻¹ · day⁻¹) from day 1 to day 21 reduced the late increases in IL-6 and MCP-1 mRNA but did not affect the rise in IL-1β mRNA on days 1 and 21 (p < 0.05).

IL-1β Up-Regulates PDE4 Activity in Human PA-SMC and Interacts with Roflumilast N-Oxide to Modulate [methyl⁻³H]Thymidine Incorporation In Vitro. Given the early rise of IL-1β mRNA in lungs after MCT exposure, as well as a report of increased plasma IL-1β in patients with severe primary PH (Humbert et al., 1995), we investigated the effect of IL-1β on PDE4 activity in human PA-SMCs. Under baseline conditions, PDE4 accounted for only 15 to 25% of total cAMP hydrolysis at 0.5 μM substrate concentration in human PA-SMCs. After 6-h incubation with IL-β (10 ng · ml⁻¹), an approximate 1.7-fold up-regulation of PDE4 activity was found that was abolished by the cyclooxygenase inhibitor indomethacin (10 μM) (Fig. 7A). Conversely, PDE4 activity was unchanged in lungs from rats given MCT (measured at several time points between 12 h and 42 days after MCT) compared with controls, accounting for approximately 30% total cAMP hydrolyzing PDE activity measured at 0.5 μM substrate concentration (data not shown).

Roflumilast N-oxide at 1 μM, which completely and selectively inhibited PDE4, did not affect serum-induced [methyl⁻³H]thymidine incorporation in human PA-SMCs. The potential of IL-1β to increase PDE4 activity prompted us to explore the effects of the PDE4 inhibitor in the presence of IL-β (10 ng · ml⁻¹). The cytokine alone reduced [methyl⁻³H]thymidine incorporation by approximately 50%, and roflumilast N-oxide synergistically diminished the remaining incorporation by another 50%. The reduction of serum-induced [methyl⁻³H]thymidine incorporation by addition of IL-1β and the PDE4 inhibitor was abolished by indomethacin (10 μM) (Fig. 7B). In the presence of IL-1β (10 ng · ml⁻¹), roflumilast N-oxide concentration-dependently reduced [methyl⁻³H]thymidine incorporation in human PA-SMCs, with IC₅₀ = 1.8 nM (Fig. 7C). Given that indomethacin abolished the inhibition of [methyl⁻³H]thymidine incorporation by roflumilast N-oxide and IL-1β, we studied the effect of the cytokine on 6-keto PGF₁α release from PA-SMCs. As expected, IL-1β increased the accumulation of the stable prostacyclin metabolite in...
culture medium after 18 h, an effect that was blocked by indomethacin (Fig. 7D). Finally, prostacyclin (1 μM) acted synergistically with the PDE4 inhibitor to significantly attenuate serum-induced \([\text{methyl}^3\text{H}]\)thymidine incorporation in human PA-SMCs (Fig. 7E).

**Discussion**

Our results show that treatment with roflumilast, a specific PDE4 inhibitor, significantly prevents and reverses the development of PH. Treatment of rats with roflumilast after MCT exposure or during continuous exposure of rats to CH was associated with smaller increases in PAP, less ventricular hypertrophy, and less vascular remodeling. Furthermore, roflumilast treatment of rats with established MCT-induced PH also significantly reversed PH and improved survival. Although PAP and right ventricular hypertrophy continued to increase from day 21 to day 42 in MCT rats given vehicle, these variables and distal vessel muscularization returned to near-normal values with roflumilast treatment.

In rats, MCT injection and chronic hypoxia are well recognized stimuli for pulmonary structural remodeling of distal pulmonary vessels and for hypertrophy and proliferation of PA-SMCs. In the group of rats that received daily roflumilast treatment, mean PAP was significantly reduced, whereas heart rate and body weight were unaltered compared with MCT control rats on day 21 after MCT injection. In addition, we found no decrease in SAP in rats treated with roflumilast (0.5 mg·kg\(^{-1}\)·day\(^{-1}\)) on day 21, in accordance with human studies showing that SAP was unaffected by roflumilast treatment (Louw et al., 2007; Nassr et al., 2007). In the MCT rat model of PH, the preventive effect of roflumilast on PH development was more marked, with 1.5 mg·kg\(^{-1}\)·day\(^{-1}\) than 0.5 mg·kg\(^{-1}\)·day\(^{-1}\). Likewise, roflumilast treatment in the group of rats exposed to chronic hypoxia significantly attenuated PH development, preventing both the hemodynamic changes and the structural remodeling. However, the higher roflumilast dose induced a mild decrease in body weight (−7%).

**Fig. 4.** Evaluation of the efficacy of roflumilast on established MCT-induced pulmonary hypertension. Roflumilast (1.5 mg·kg\(^{-1}\)·day\(^{-1}\)) or vehicle was administered to Wistar rats from day 21 to day 42 after MCT. Pulmonary artery pressure (A), RV/LV+S ratio (B), and muscularization of intraacinar pulmonary vessels (C) were assessed on days 21 and 42. Data are means ± S.E.M. from at least 13 rats per group (except pulmonary artery pressure, which was obtained from five rats per group). *, \(p < 0.05\); **, \(p < 0.01\); and ***, \(p < 0.001\) compared with day 42 after MCT in rats treated with vehicle (Dunn’s test after Kruskal-Wallis test). NM, nonmuscularized; PM, partially muscularized; and FM, fully muscularized.

**Fig. 5.** Evaluation of the efficacy of roflumilast on pulmonary vascular remodeling induced by MCT. Representative photomicrographs of pulmonary arterioles on day 21 after MCT (A and B), on day 42 after MCT with vehicle (from day 21 to day 42) (C and D), and on day 42 after MCT with roflumilast (1.5 mg·kg\(^{-1}\)·day\(^{-1}\) from day 21 to day 42) (E and F). Media hypertrophy was associated with increased numbers of proliferating vascular smooth muscle cells, as shown by immunohistochemistry for PCNA; brown nuclei are PCNA-positive cells. Magnifications: 25× (A, C, and E) and 40× (B, D, and F).
In a previous study, rolipram, another PDE4 inhibitor, attenuated acute hypoxic vasoconstriction in isolated perfused rat lung (Phillips et al., 2005) but had little effect on PH development or vascular remodeling in response to CH. The discrepancy between this study of rolipram and our study of roflumilast is probably explained by differences in potencies (Hatzelmann and Schudt, 2001), administration routes, and dosing resulting in different degrees of PDE4 inhibition by these two drugs. That roflumilast exerts a beneficial effect in a model of CH induced PH is an important finding.

In our present study that more specifically addressed the role for PDE4 in the development of MCT-induced PH, we measured PDE4 activity in lung tissue from rats at baseline and various times after MCT administration. Compared with control rats, PDE4 activity did not significantly change during development and after establishment of PH (data not shown). In a previous study investigating PDE4 in chronic hypoxia-induced PH, no change in activity was found in pulmonary arteries from hypoxic rats compared with normoxic animals, except for a small decrease in the resistance arteries (Maclean et al., 1997). In a more recent study, measurement of PDE activity in SMCs from rat pulmonary arteries showed that PDE3 and PDE4 were the predominant isoforms responsible for cAMP hydrolysis in these cells (Phillips et al., 2005). Previous studies investigating the role of PDE in hypertensive pulmonary vasculature found that PDE activity and PDE3 gene transcript were increased in pulmonary arteries from rats with CH-induced PH (Maclean et al., 1997; Wagner et al., 1997) and that inhibition of PDE3 and PDE4 activities significantly improved PH. Intravenous infusion of the dual selective PDE3/4 inhibitor tolafentrine, when given chronically, causes dose-dependent vasodilation and attenuation of right ventricular hypertrophy and pulmonary vascular remodeling. Moreover, reversal of established PH was seen with iloprost and tolafentrine infused in combination (Schermuly et al., 2004).

Despite the lack of an increase in PDE4 activity during the development of MCT-induced PH, roflumilast not only significantly attenuated the development of MCT-induced PH but also reversed established PH. After 3 weeks of treatment, PAP on day 42 was not only lower than the value in the MCT-group treated with vehicle but also was lower than the value on day 21 before the therapeutic intervention. Concomitant marked decreases in right ventricular hypertrophy and pulmonary vascular remodeling were noted. Roflumilast treatment from day 21 to day 42 almost completely reversed distal vessel muscularization and medial wall hypertrophy in the pulmonary arteries. Moreover, whereas cell proliferation assessed by PCNA labeling was observed in the pulmonary vessel wall on day 21 and was even more marked on day 42 in vehicle-treated rats, roflumilast treatment was associated with a reduction in PCNA labeling on day 42. It is therefore likely that roflumilast reversed PH by inhibiting the marked proliferation of cells that was required for the maintenance and late aggravation of PH.

Nevertheless, we cannot exclude that attenuation and reversal of PH by PDE4 inhibition was partly related to anti-inflammatory effects. Recent experimental data in the MCT model of PH suggest that proinflammatory cytokines and chemokines may play a role in PA-SMC proliferation (Guignabert et al., 2005). In the present study and in a previous study (Guignabert et al., 2005), we observed an early increase in IL-1β mRNA preceding the development of MCT-induced PH. Although roflumilast did not affect the early or late increases in IL-1β mRNA, it markedly attenuated the late rises in MCP-1 and IL-6 mRNA. However, expression of these cytokines was concomitant with PH development and was therefore possibly a consequence rather than a cause of PH. This possibility is supported by the fact that 5-hydroxytryptamine transporter blockade, which reverses PH (an effect related to the role of the 5-hydroxytryptamine transporter in the pathogenesis of smooth muscle proliferation), simultaneously normalizes the late expression of these cytokines (Guignabert et al., 2005).

Human PA-SMCs incubation with IL-1β was associated with an increase in PDE4 activity. Endogenous prostacyclin induced by IL-1β possibly accounted for this finding, because the increment in PDE4 activity was abolished by the cyclooxygenase inhibitor indomethacin. The cAMP increase in response to prostacyclin is well known to up-regulate PDE4 and late aggravation of PH.

MCP-1 mRNA concentrations in lungs from MCT-treated rats. Rats received MCT (60 mg kg⁻¹ s.c., single dose) and were treated with roflumilast (1.5 mg kg⁻¹ day⁻¹) or vehicle for 21 days. IL-1β, IL-6, and MCP-1 mRNA in rat lung extracts were quantified by real-time reverse transcription-PCR before and 1 and 21 days after MCT exposure. Relative mRNA expression of the target gene compared with 18S RNA is reported as the mean ± S.E.M. from 5 rats. *p < 0.05 compared with MCT and vehicle.
roflumilast N-oxide concentration-dependently reduced \([^3]H\)thymidine incorporation to a maximum efficacy of 50 to 60% and with an IC\(_{50}\) value close to half-maximal inhibition of PDE4 activity (Hatzelmann and Schudt, 2001). This effect was reversed by indomethacin, supporting the possibility that IL-1\(\beta\)-induced release of prostanoids (which increase cAMP formation) may enable the PDE4 inhibitor to inhibit DNA synthesis. Indeed, in the presence of PGI\(_2\), PDE4 inhibition significantly decreased \([^3]H\)thymidine incorporation. However, the reduction of DNA synthesis by PGI\(_2\) (1 \(\mu\)M) was small compared with the effect of IL-1\(\beta\), indicating that other mediators may support the effects of this cytokine. One fact that may help to reconcile previous findings where roflumilast on its own inhibited DNA synthesis (Growcott et al., 2006) with the findings from the present study is that different mitogens (platelet-derived growth factor or serum) or culture conditions might result in different levels of cAMP synthesis contingent on endogenous agonists such as prostanoids. Conceivably, in vivo MCT-induced IL-1\(\beta\) expression may support the inhibition by roflumilast of pulmonary vessel muscularization.

In conclusion, the PDE4 inhibitor roflumilast significantly attenuates pulmonary vascular remodeling and hypertension induced by chronic hypoxia or MCT in both a preventive (MCT and hypoxia) and a therapeutic (MCT) protocol. These observations may be attributed to an integrated network of cellular effects of the PDE4 inhibitor in conjunction with endogenous prostanoids on recruitment, proliferation, and contractile tone of pulmonary vascular smooth muscle cells and inflammatory cells. The precise role of the PDE4 inhibitor on each of these cell populations remains to be explored.

**Fig. 7.** IL-1\(\beta\) up-regulates PDE4 activity and interacts with roflumilast N-oxide on \([^3]H\)thymidine incorporation in human PA-SMCs. Human PA-SMCs were preincubated with vehicle (DMSO) or indomethacin (INDO) and cultured with IL-1\(\beta\) (10 ng \(\cdot\) ml\(^{-1}\)) for 6 h. PDE4 activity (picomoles per minute per milligram of protein) was measured in cell lysates at 0.5 \(\mu\)M cAMP. ++, \(p < 0.01\) versus control; +, \(p < 0.01\) versus IL-1\(\beta\) and vehicle (DMSO) (A). To assess the effect of roflumilast N-oxide (RNO; 1 \(\mu\)M), IL-1\(\beta\) (10 ng \(\cdot\) ml\(^{-1}\)), INDO (10 \(\mu\)M), or vehicle (DMSO) on cell proliferation, human PA-SMCs were preincubated with these drugs and then stimulated with 5% FBS for 24 h. Effect of 5% FBS was defined as 100% cpm/well incorporated. ++, \(p < 0.01\) versus IL-1\(\beta\) and vehicle (B). PA-SMCs were preincubated with roflumilast N-oxide (10 \(\mu\)M), vehicle (DMSO), and IL-1\(\beta\) (10 ng \(\cdot\) ml\(^{-1}\)) and stimulated with 5% FBS. \([^3]H\)Thymidine incorporation was measured as described in text. Percentage of inhibition of \([^3]H\)thymidine incorporation in the presence of IL-1\(\beta\) was calculated for each concentration of the PDE4 inhibitor (C). Growth-arrested human PA-SMCs were preincubated with vehicle (DMSO) or indomethacin and cultured with IL-1\(\beta\) (10 ng \(\cdot\) ml\(^{-1}\)) for 18 h. 6-Keto PGF\(_{1\alpha}\) (picograms per milliliter) was measured in culture supernatants (D). Human PA-SMCs were preincubated with vehicle (DMSO), RNO (1 \(\mu\)M), PGI\(_2\) (1 \(\mu\)M), or PGI\(_2\) and RNO (1 \(\mu\)M), and then stimulated with 5% FBS for 24 h. ++, \(p < 0.05\) versus vehicle alone; *, \(p < 0.01\) versus PGI\(_2\) alone (E). Results are shown as the mean \(\pm\) S.E.M. from \(n = 3\) (A and D), \(n = 4\) (B and C), or \(n = 5\) (E) experiments.
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