Phosphodiesterase 4 inhibition attenuates pulmonary inflammation in neonatal lung injury

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Abstract

Phosphodiesterase-4 (PDE4) inhibitors may offer novel therapeutic strategies in respiratory disease, including asthma and chronic obstructive pulmonary disease. Therefore, selective PDE4 inhibitors may also provide a therapeutic option for very preterm infants with bronchopulmonary dysplasia (BPD). We investigated the anti-inflammatory effect of two PDE4 inhibitors in a preterm rat model of hyperoxia-induced lung injury.

Preterm rat pups were exposed to room air, hyperoxia or hyperoxia and one of two PDE4 inhibitors: rolipram and piclamilast. The anti-inflammatory effects of prolonged PDE4 inhibitor therapy were investigated by studying survival, histopathology, fibrin deposition, alveolar vascular leakage and differential mRNA expression (real-time RT-PCR) of key genes involved in inflammation, alveolar enlargement, coagulation and fibrinolysis.

PDE4 inhibitor therapy prolonged median survival up to 7 days, reduced alveolar fibrin deposition, lung inflammation, and vascular leakage by decreasing the influx of monocytes and macrophages and protein efflux in bronchoalveolar lavage fluid. Analysis of mRNA expression of key genes involved in experimental BPD revealed a significant PDE4 inhibitor-induced improvement of genes involved in inflammation, fibrin deposition and alveolarization.

We conclude that PDE4 inhibition prolongs survival by inhibiting inflammation and reducing alveolar fibrin deposition in preterm rat pups with neonatal hyperoxic lung injury, whereby piclamilast outperformed rolipram.
Key words: bronchopulmonary dysplasia, coagulation, fibrin deposition, oxidative stress, piclamilast, rolipram.
Introduction

Pharmacological and technical advances in neonatal intensive care medicine have greatly improved the survival and morbidity of premature infants. The preterm lung is highly susceptible to injury during resuscitation and mechanical ventilation and to pro-inflammatory mediators interfering with signalling required for normal late gestational lung development (1). Pro-inflammatory mediators may be elevated because of fetal or postnatal infection or by release from preterm lungs ventilated at either (too) low or high lung volumes. Preterm infants of <30 weeks of gestation and a birth weight of <1,200 g are at high risk for perinatal lung injury, which can progress to chronic lung disease (bronchopulmonary dysplasia, BPD). BPD is characterized by an arrest in alveolar and vascular lung development, complicated by inflammation, abnormal coagulation and fibrinolysis with intra-alveolar fibrin accumulation, oxidative stress, and at later stages by pulmonary hypertension (1, 2).

Treatment of BPD with glucocorticoids has been refuted because of a higher incidence of neurological morbidity in long-term survivors. Theophylline, a non-selective phosphodiesterase (PDE) inhibitor, is widely used in neonatal intensive care to treat apnea of prematurity and wean preterm infants at risk for developing BPD from the ventilator because it increases respiratory drive and has an immunomodulatory effect (3, 4). Since inflammation and unbalanced coagulation and fibrinolysis, leading to extravascular fibrin deposition in the lung, are two interrelated processes that play a pivotal role in the pathophysiology of inflammatory lung disease, we investigated whether the development of BPD can be interrupted by intervening in the vicious cycle of inflammation and coagulation. We have previously shown that the methylxanthine derivative pentoxifylline reduces alveolar fibrin deposition and vascular alveolar leakage, and prolongs survival in rats with neonatal hyperoxic lung injury (5), a suitable in vivo
model for experimental BPD (6). Pentoxifylline can exert its protective effect in inflammatory lung diseases in different ways, including inhibition of vascular leakage, improved vascular blood flow by reducing blood viscosity and improved red blood cell flexibility by increasing membrane fluidity, and inhibition of leukocyte activation (7-9). The protective effects in inflammatory lung diseases have been partially ascribed to the weak non-selective inhibition of PDEs by pentoxifylline, resulting in increased intracellular cAMP levels (10, 11). PDEs belong to an enzyme family with 11 different members, iso-enzymes PDE1-11, which exert their biological function by inactivating the intracellular messengers cAMP and/or cGMP by hydrolysis (12-14). PDE4 is a cAMP-specific phosphodiesterase that consists of 4 sub-families (PDE4A-D) with various splice variants encoding at least 35 different PDE4 proteins. PDE4 isoforms are expressed selectively in brain, leukocytes (including neutrophilic and eosinophilic granulocytes, macrophages and T-lymphocytes), mast cells, dendritic cells and in the vascular endothelium (15). Inhibition of PDE4 has anti-inflammatory properties in inflammatory pulmonary diseases in adult laboratory animals (16-19) and has therapeutic potential in patients suffering from chronic obstructive lung disease (COPD), acute respiratory distress syndrome (ARDS) and asthma (13, 14, 20-24). BPD and COPD are serious chronic lung diseases at the extreme stages of life and PDE4 inhibitors may also provide a therapeutic option for very premature infants with BPD. We investigated the anti-inflammatory properties of rolipram, which is the specific prototypic PDE4 inhibitor (13, 14, 16) and piclamilast, which is a second generation PDE4 inhibitor (14), in an animal model for experimental BPD, in which chronic lung disease is induced in premature rats by prolonged exposure to 100% oxygen. We found that PDE4 inhibition has anti-inflammatory properties, attenuates pulmonary fibrin deposition and vascular-alveolar leakage, and prolongs survival in hyperoxia-induced neonatal lung injury.
**Materials and Methods**

*Animals*

Timed-pregnant Wistar rats were kept in a 12 h dark/light cycle and fed a standard chow diet (Special Diet Services, Witham, Essex, England) *ad libitum*. Breeding pairs were allowed access for one hour on the day female rats showed very specific sexual behaviour: lordosis, hopping and air-flapping. After a gestation of approximately 21 1/2 days pregnant rats were killed by decapitation (spontaneous birth occurs 22 days after conception) and pups were delivered by hysterectomy through a median abdominal incision to ensure that the delay in birth between the first and the last pup is only 5 minutes. Immediately after birth, pups were dried and stimulated. Pups from four litters were pooled and distributed over two experimental groups: an oxygen (O₂) group and an oxygen-PDE4 inhibitor (rolipram or piclamilast) group, and room air-exposed (RA) control groups. Litter size was 12 pups per litter in the experimental groups. Pups were kept in a transparent 50 x 50 x 70 cm Plexiglas chamber for 10 days or until death occurred (survival experiments). In this way influences of the birth process within and between litters can be avoided and exposure to hyperoxia can be started within 30 minutes after birth. Pups were fed by lactating foster dams, which were rotated daily to avoid oxygen toxicity. Foster dams were exposed to 100% oxygen for 24 hours at 72 hours intervals and to room air for 48 hours. The oxygen concentration was kept at 100% using a flow of 2.5 L/min. Oxygen concentrations were monitored daily with an oxygen sensor (Drägerwerk AG, Lübeck, Germany). Weight, evidence of disease, and mortality were also checked daily. Hyperoxia exposed pups were injected subcutaneously, starting on day 2, via a 0.5 ml syringe (U-100 Micro-Fine insulin 29G syringe, Becton Dickinson, NY, USA) at the lower back. In the PDE4 inhibitor experiments pups received either 150 µl rolipram (racemic rolipram R6520, Sigma, St. Louis, MO, USA) in 0.9%
saline (containing 0.1-0.5% ethanol) or 150 µl 0.9% saline (containing 0.1-0.5% ethanol) in age-matched oxygen- and room air-exposed controls. In the piclamilast experiments pups received either 150 µl piclamilast (a gift from Altana Pharma AG, Konstanz, Germany) in 0.9% saline (containing 0.05-0.1% DMSO) or 150 µl 0.9% saline (containing 0.05-0.1% DMSO) in age-matched oxygen- and room air-exposed controls. In a pilot experiment in which rats were treated with 125, 250, 500, 1000 and 2500 µg/kg/day rolipram under hyperoxia, we found that pups, continuously exposed to 100% O₂ and 1000 and 2500 µg/kg/day rolipram were not fed properly by the foster dams and lost up to 25% of their initial body weight after 4 days. These pups were killed on day 4 because of starvation. Pups treated with 500 µg/kg/day rolipram failed to gain weight and only 33% of them survived for 10 days. Pups treated with 125 and 250 µg/kg/day rolipram survived the experimental period of 10 days. Therefore, experiments were performed with 125 and 250 µg/kg/day rolipram. In a second pilot experiment in which rats were treated with 2.5, 5.0, 10 and 20 mg/kg/day piclamilast under hyperoxia we found that rat pups treated with 10 and 20 mg/kg/day were poorly fed by the foster dams, judged by visual inspection of their stomach content and growth. Therefore, experiments were performed with 2.5 and 5.0 mg/kg/day piclamilast. Results obtained with both PDE4 inhibitors are presented relative to their own oxygen- and room air-exposed controls to prevent the use of historical controls and because controls were treated with a different solvent containing trace amounts of ethanol for rolipram and trace amounts of DMSO for piclamilast. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Leiden University Medical Center and were performed according to the Helsinki convention for the use and care of animals.

*Tissue preparation*
Pups were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg body weight; Nimatek, Eurovet Animal Health BV., Bladel, The Netherlands) and xylazine (50 mg/kg body weight; Rompun, Bayer, Leverkusen, Germany). To avoid postmortem fibrin deposition in the lungs, heparin (100 units; Leo Pharma, Breda, The Netherlands) was injected intraperitoneally. After 5 min, pups were exsanguinated by transection of the abdominal blood vessels. The thoracic cavity was opened, and the lungs were removed, snap-frozen in liquid nitrogen, and stored at –80°C until use for real-time RT-PCR or the fibrin deposition assay. For histology studies, the trachea was cannulated (Bioflow 0.6 mm intravenous catheter, Vygon, Veenendaal, The Netherlands), and the lungs were fixed in situ via the trachea cannula with buffered formaldehyde (4% paraformaldehyde in PBS, pH 7.4) at 25 cm H₂O pressure for 3 min. Lungs were removed, fixed additionally in formaldehyde for 24 h at 4°C, and embedded in paraffin after dehydration in a graded alcohol series and xylene.

**Bronchoalveolar lavages**

Pups were anesthetized with an intraperitoneal injection of ketamine and xylazine and injected intraperitoneally with heparin. A cannula (Bioflow 0.6 mm intravenous catheter) was positioned in the trachea, and the pups were exsanguinated by transection of the abdominal blood vessels. Lungs were slowly lavaged two times with 500 µl 0.15 M NaCl, 1 mM EDTA (pH 8.0), without opening the thorax. Samples were pooled, stored temporarily at 4°C and centrifuged for 10 min at 5,000 rpm. Supernatants were stored at -20 °C until further use.

**Lung histology**
Lung paraffin sections (5 µm) were cut and mounted onto SuperFrost plus-coated slides (Menzel, Braunschweig, Germany). After deparaffinization, sections were stained with hematoxylin and eosin or with monoclonal ED-1 that specifically recognizes rat monocytes and macrophages (25) or with monoclonal 59D8 that specifically recognizes rat β-fibrin (6). For immunohistochemistry, sections were incubated with 0.3% H₂O₂ in methanol to block endogenous peroxidase activity. After a graded alcohol series, sections were boiled in 0.01 M sodium citrate (pH 6.0) for 10 min. Sections were incubated overnight with monoclonal ED-1 or 59D8, stained with EnVision-HRP (Dako, Glostrup, Denmark), using NovaRed (Vector, Burlingame, CA) as chromogenic substrate, and counterstained briefly with hematoxylin. For morphometry, an eye piece reticle with a coherent system of 21 lines and 42 points (Weibel type II ocular micrometer; Paes, Zoeterwoude, The Netherlands) was used. Mean linear intercept (MLI), an indicator of mean alveolar diameter, was assessed in 10 non-overlapping fields at a 200x magnification in one section for each animal. The density of ED-1 positive monocytes and macrophages was determined by counting the number of cells per. Fields containing large blood vessels or bronchioli were excluded from the analysis. Results were expressed as relative number of cells per mm². Per experimental animal 22 fields in one section were studied at a 400x magnification. At least 6 different rat pups per experimental group were studied.

Fibrin detection assay

Fibrin deposition in lungs was detected as described previously (6). Briefly, frozen lungs were homogenized with an Ultra-Turrax T25 basic tissue homogenizer (IKA-Werke, Staufen, Germany) for 1 min at full speed in a cold 10 mM sodium phosphate buffer (pH 7.5), containing 5 mM EDTA, 100 mM ε-aminocaproic acid, 10 U/ml aprotinin, 10 U/ml heparin, and 2 mM
phenylmethanesulfonyl fluoride. The homogenate was incubated for 16 h on a top over top rotor at 4°C. After centrifugation (10,000 rpm, 4°C, 10 min), the pellet was resuspended in extraction buffer [10 mM sodium phosphate buffer (pH 7.5), 5 mM EDTA, and 100 mM ε-aminocaproic acid] and recentrifuged. Pellets were suspended in 3 M urea, extracted for 2 h at 37°C, and centrifuged at 14,000 rpm for 15 min. After the supernatant was aspirated and discarded, the pellet was dissolved at 65°C in reducing sample buffer (10 mM Tris pH 7.5, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.4 mg/ml bromophenol blue) for 90 min in a thermomixer (Eppendorf, Hamburg, Germany) with continuous mixing at 900 r.p.m. Hereafter, samples were subjected to SDS-PAGE (7.5%; 5% stacking) and blotted onto PVDF membrane (Immobilon-P, Millipore, Bredford, MA). The 56-kDa fibrin β-chains were detected with monoclonal 59D8, which specifically recognizes β-fibrin (6, 26), using ECL plus Western blotting detection system and Hyperfilm ECL (Amersham Biosciences, Arlington Heights, IL). Exposures were quantified with a Bio-Rad GS-800 calibrated densitometer using the Quantity One, version 4.4.1 software package (Bio-Rad, Veenendaal, the Netherlands). Fibrin deposition was quantified in lungs of at least ten rats per experimental group. As a reference, fibrin standards were generated from rat fibrinogen (Sigma, St. Louis, MO). After rat fibrinogen was solubilized in two-thirds strength PBS (pH 7.4), human α-thrombin (Sigma, St. Louis, MO) was added, vortexed, and incubated at 37°C for 10 min. After addition of 2x SDS sample buffer, the fibrin sample was vortexed and incubated at 65°C for 90 min; aliquots were frozen at –80°C until use.

**Real-time RT-PCR**

Total RNA was isolated from lung tissue homogenates using guanidium-phenol-chloroform extraction and isopropanol precipitation (RNA-Bee, Tel-Test inc., Bio-Connect BV,
Huissen, the Netherlands). The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically. The integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide. Samples did not show degradation of ribosomal RNA by visual inspection under ultraviolet light. First-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, the Netherlands) by mixing 2 µg total RNA with 0.5 µg of oligo(dT)12-18 primer in a total volume of 12 µl. After the mixture was heated at 70°C for 10 min, a solution containing 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM dNTPs, 0.5 µl RNase inhibitor, and 200 U Superscript Reverse Transcriptase was added, resulting in a total volume of 20.5 µl. This mixture was incubated at 42°C for 1 h; total volume was adjusted to 100 µl with RNase-free water and stored at –80°C until further use. For real-time quantitative PCR, 1 µl of first-strand cDNA diluted 1:10 in RNase-free water was used in a total volume of 25 µl, containing 12.5 µl 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 200 ng of each primer. Primers, designed with the Primer Express software package (Applied Biosystems), are listed in Table 1. PCR reactions, consisting of 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles), were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems) of the Leiden Genome Technology Center. Data were analyzed with the ABI Prism 7900 sequence detection system software (version 2.2.2) and quantified with the comparative threshold cycle method with β-actin as a housekeeping gene reference (27).

Protein assay

Total protein concentration was measured in bronchoalveolar lavage fluid (BALF) using the Dc protein assay (Bio-Rad, Veenendaal, the Netherlands), according to the manufacturer’s
instructions with bovine serum albumin, fraction V (Roche Diagnostics GmbH, Mannheim, Germany) as a standard. The detection limit was 31 µg/ml.

Statistical analysis

Values are expressed as mean ± SEM. Differences between groups (≥ 3) were analyzed with analysis of variance (ANOVA), followed by Tukey’s multiple comparison test or with Student’s t-test between 2 groups. For comparison of survival curves, Kaplan-Meier analysis followed by a log rank test was performed. Differences at p values < 0.05 were considered statistically significant.
Results

Fibrin deposition

Fibrin deposition, a sensitive marker for tissue damage in hyperoxia-induced lung injury, was studied in lung homogenates by Western blot analysis using the monoclonal 59D8 antibody against the 56 kDa fibrin β-chain (Figure 1A) and was used as a readout for our intervention studies. Fibrin deposition was quantified (Figure 1B) after treatment with two different concentrations of the PDE4 inhibitors rolipram (125 and 250 µg/kg/day) or piclamilast (2.5 and 5.0 mg/kg/day). Fibrin deposition was at background levels during normal neonatal pulmonary development on day 10 (<25 ng fibrin/mg tissue). Fibrin deposition increased more than 10-fold to 357.9 ± 54.3 ng fibrin/mg tissue in lungs of pups exposed to 100% oxygen for 10 days (p < 0.001) in the rolipram group and to 258.4 ± 53.7 ng fibrin/mg tissue (p < 0.001) in the piclamilast group. PDE4 inhibitor treatment attenuated fibrin deposition up to 2.5-fold to 145.5 ± 19.8 (p < 0.01) ng fibrin/mg tissue for 250 µg/kg/day of rolipram, and up to 6-fold to 43.2 ± 7.9 (p < 0.05) for 5.0 mg/kg/day of piclamilast compared to their oxygen-exposed controls. In paraffin sections extravascular fibrin deposits were detected in septa and alveoli after exposure to hyperoxia (Figure 1D), but were minor or even absent in pups treated with 5.0 mg/kg/day piclamilast (Figure 1E). Pulmonary fibrin deposition was absent in normoxia (Figure 1C). Similar results were obtained in rolipram treated pups (data not shown).

The most effective piclamilast dosage was 5.0 mg/kg/day. The difference in fibrin deposition between 125 and 250 µg/kg/day of rolipram and oxygen-exposed controls was rather small, 2.1 and 2.5-fold, respectively, but in combination with the survival data 250 µg/kg/day of rolipram was the most effective dosage. Additional experiments were performed with the most effective dosage of each PDE4 inhibitor. Because quantitative RT-PCR and fibrin deposition
could be determined in the same experimental pups, we included both concentrations of each PDE4 inhibitor in the *real-time* RT-PCR studies.

**Growth and survival**

At birth, on postnatal day 1, mean body weight of the preterm rat pups was 4.6 g (Figure 2, panels A and C). Growth of pups treated with 125 µg/kg/day of rolipram was not different from oxygen-exposed controls (Figure 2A). In pups treated with 250 µg/kg/day of rolipram or 5.0 mg/kg/day of piclamilast (Figure 2C) growth was significantly retarded from day 7 onward compared to oxygen-exposed controls (*p* < 0.05): mean weight was 8.7 g in both PDE4 inhibitor-treated groups versus 13.9 g in oxygen-exposed pups on day 10. Survival of oxygen-exposed pups treated with both PDE4 inhibitors was prolonged significantly. Rolipram prolonged survival in a concentration-dependent way compared to oxygen-exposed controls (Figure 2B; 125 µg/kg/day: *p* = 0.04 and 250 µg/kg/day: *p* < 0.001): median survival of oxygen-exposed controls was 11 days and of oxygen-exposed pups treated with 125 and 250 µg/kg/day of rolipram 14 and 17 days, respectively (Figure 2B). In the piclamilast group median survival of oxygen-exposed controls was 12 days and piclamilast (5.0 mg/kg/day) prolonged median survival with 7 days compared to oxygen-exposed controls (Figure 2D; *p* < 0.001). Pups in room air did not show signs of illness or mortality during the first 4 weeks after birth (data not shown).

**Lung histology**

Preterm rats are born at the saccular stage of lung development. Sacculi are transformed into alveoli in the second week after birth by septal thinning and secondary septation (Figure 3A). Oxygen exposure for 10 days resulted in edema and a heterogeneous distribution of
enlarged air-spaces, which were surrounded by septa with a marked increase in septal thickness (Figure 3B). PDE4 inhibitors improved lung histopathology during hyperoxia exposure by thinning of septa, reducing inflammation and decreasing edema, resulting in enlarged alveoli (Figure 3, panels C and D). Hyperoxia led to a massive inflammatory reaction, characterized by an overwhelming influx of inflammatory cells (Figure 3B), including macrophages and neutrophils. Macrophages were detected with monoclonal ED-1 (Figure 3A-D) and quantified by morphometry (Figure 3E). Resident ED-1-positive monocytes and macrophages were present at 350 cells per mm$^2$ in septa and alveoli of control lungs (Figure 3A), whereas lungs of oxygen-exposed pups contained 4 times as many (Figure 3B). PDE4 inhibitor treatment reduced the influx of ED-1 positive cells 2.4-fold ($p = 0.02$) and 1.8-fold ($p < 0.001$) for rolipram and piclamilast, respectively, compared to oxygen-exposed controls.

Mean linear intercept increased by 20% after exposure to hyperoxia compared to room air-exposed controls ($p = 0.01$) and did not improve significantly after rolipram or piclamilast treatment (Figure 3F), indicating that PDE4 inhibitor treatment did not affect the hyperoxia-induced increase in alveolar size (Figure 3, panels C and D).

**Protein in bronchoalveolar lavage fluid**

Total protein concentration in bronchoalveolar lavage fluid (BALF) was measured to establish the inhibitory effect of PDE4 inhibitor treatment on pulmonary edema by capillary-alveolar leakage (Figure 4). Protein concentration on postnatal day 10 increased 5-10-fold after hyperoxia, and reduced 2.7-fold ($p < 0.05$) or 9.5-fold ($p < 0.01$) after treatment with rolipram or piclamilast, respectively.
mRNA expression in lung homogenates

Hyperoxia-induced lung injury induces alterations in inflammation, coagulation, fibrinolysis, alveolar enlargement, and edema. Therefore, we studied differential expression of key genes of these pathways, previously characterized in this rat model for experimental BPD (6), with real-time RT-PCR in lungs of pups exposed to room air, 100% oxygen, or 100% oxygen with PDE4 inhibitor (125 or 250 µg/kg/day of rolipram and 2.5 or 5.0 mg/kg/day of piclamilast) on postnatal day 10 to characterize the optimal response to both PDE4 inhibitors in hyperoxia-induced lung injury. Ten days of oxygen exposure resulted in an increase in mRNA expression of the pro-inflammatory markers (Figure 5) IL-6 (100-fold; \( p < 0.001 \)), the chemokines chemokine-induced neutrophilic chemoattractant-1 (CINC-1, 10-fold; \( p < 0.001 \)) and monocyte chemoattractant protein 1 (MCP-1, 11-fold; \( p < 0.001 \)), the growth factor amphiregulin (6-fold; \( p < 0.001 \)), the coagulant factors (Figure 6) tissue factor (TF, 3-fold; \( p < 0.0001 \)) and endothelial protein c receptor (EPCR, 7-fold; \( p < 0.001 \)), the fibrinolytic factors (Figure 7) plasminogen activator (PA) inhibitor 1 (PAI-1; 40-fold; \( p < 0.001 \)), tissue type PA (tPA; 1.3-fold; \( p < 0.001 \)), urokinase PA (uPA; 1.4-fold; \( p < 0.001 \)) and uPA receptor (uPAR; 6-fold; \( p < 0.001 \)) and a decrease in the expression of genes related to alveolar enlargement (Figure 8) vascular endothelial growth factor receptor 2 (VEGFR2, 4-fold; \( p < 0.001 \)) and fibroblast growth factor receptor 4 (FGFR4, 8-fold; \( p < 0.001 \)) in lungs of oxygen-exposed pups compared to room air controls.

Treatment of pups with 125 µg/kg/day of rolipram decreased mRNA expression of amphiregulin 2.4-fold (\( p < 0.01 \); Figure 5D), tPA 1.4-fold (\( p < 0.001 \); Figure 7B) and uPAR 1.5-fold (\( p < 0.01 \); Figure 7D), and increased the expression of VEGFR2 1.5-fold (\( p < 0.01 \); Figure 8A) compared to oxygen-exposed controls. In pups treated with 250 µg/kg/day of rolipram the
decrease in mRNA expression was 2.6-fold ($p < 0.05$), 1.7-fold ($p < 0.05$), 1.6-fold ($p < 0.001$) and 1.7-fold ($p < 0.001$) for IL6 (Figure 5A), CINC-1 (Figure 5B), tPA (Figure 7B) and uPAR (Figure 7D) respectively, compared to oxygen-exposed controls. Expression of VEGFR2 mRNA was increased 1.6-fold ($p < 0.001$) in pups treated with 250 µg/kg/day of rolipram compared to oxygen-exposed controls (Figure 8A), whereas expression of the other genes studied were not significantly different from oxygen-exposed controls.

Treatment of pups with 2.5 mg/kg/day of piclamilast decreased mRNA expression of uPAR 1.5-fold ($p < 0.05$; Figure 7D) and increased the expression of VEGFR2 1.9-fold ($p < 0.001$; Figure 8A) and FGFR4 2.0-fold ($p < 0.001$; Figure 8B) compared to oxygen-exposed controls. In pups treated with 5.0 mg/kg/day of piclamilast mRNA expression was decreased for the inflammatory genes (Figure 5): IL6 (5.8-fold; $p < 0.001$), CINC-1 (2.4-fold; $p < 0.001$), MCP-1 (3.9-fold; $p < 0.001$) and amphiregulin (3.6-fold; $p < 0.001$) and genes involved in coagulation (Figure 6) and fibrinolysis (Figure 7): TF (2.0-fold; $p < 0.001$), EPCR (1.7-fold; $p < 0.001$), PAI-1 (1.5-fold; $p < 0.001$), tPA (1.6-fold; $p < 0.001$) and uPAR (1.9-fold; $p < 0.001$), and increased in genes involved in alveolar enlargement (Figure 8): VEGFR2 (1.9-fold; $p < 0.001$) and FGFR4 (1.9-fold; $p < 0.05$) compared to oxygen-exposed controls.
Discussion

PDE4 inhibitor therapy with rolipram and piclamilast prolonged survival of premature rat pups exposed to prolonged hyperoxia by inhibiting inflammation, reducing capillary-alveolar protein leakage and alveolar septum thickness, and attenuating alveolar fibrin deposition. The positive effect of PDE4 inhibition on inflammation is secondary to a reduced expression of inflammatory genes, including IL6 and CINC-1, decreased influx of neutrophils and macrophages into the lung and a lower protein content in bronchoalveolar lavage fluid, a marker for capillary-alveolar leakage. These findings are in agreement with observations in rats, mice and guinea pigs that PDE4 inhibition reduces the inflammatory response in various models of acute pulmonary inflammation, including LPS- and antigen-induced lung injury (17, 19, 28-30). The importance of PDE4 inhibition as a therapeutic strategy in inflammatory lung diseases, induced by oxidative stress, is further supported by in vitro studies, in which PDE4D isoforms are activated in macrophages stimulated with H2O2 (31). In addition, low oxygen concentrations upregulate PDE4 isoforms and intracellular cAMP levels in human pulmonary artery smooth muscle cells (32) and may contribute to the development of pulmonary arterial hypertension, a complication of BPD (2). Because hypoxia and hyperoxia show an overlap in gene expression changes in some of the target genes (33), PDE4 isoform expression may also be differentially altered in hyperoxia-induced experimental BPD and play a role in the development of neonatal chronic lung disease.

Treatment with rolipram or piclamilast effectively reduces fibrin deposition in the lungs of hyperoxia-exposed premature rat pups. Fibrin deposition is a sensitive marker for lung tissue damage and an important contributor to the pathogenesis of pulmonary injury by oxidative stress. In both human and animal lung injury, intra-alveolar and intravascular fibrin deposition is
correlated with a poor prognosis and reduction of fibrin deposition contributes to a better outcome in experimental lung injury in rodents and baboons (5, 34, 35). Fibrin induces lung injury in various ways. It exerts pro-inflammatory and pro-fibrotic properties by facilitating cell migration and activating inflammatory cells and fibroblasts, probably via activation of the transcription factor nuclear factor-κB and AP-1 proteins (36), and can hamper pulmonary gas exchange via inactivation of lung surfactant (37). Reduced pulmonary fibrin deposition by PDE4 inhibitor treatment can, at least in part, be explained by a reduction in the expression of key genes involved in coagulation and fibrinolysis: tissue factor, plasminogen activator (PA) inhibitor I and tPA for piclamilast, and tPA for rolipram. The somewhat different effects of the PDE4 inhibitors on hyperoxia-induced changes of gene expression profiles probably reflect that piclamilast may be more effective than rolipram in reducing inflammation and fibrin deposition at doses that do not cause adverse effects. A direct effect of PDE4 inhibition on fibrin deposition is suggested by findings of Sachs et al (38) who demonstrated that reduced fibrinolysis in lung fibrosis by p75 neurotrophin receptor is mediated through inhibition of plasminogen activation via PDE4-enhanced cAMP degradation. Fibrin is primarily deposited in the extravascular compartment, including septa and alveolar lumen (5), indicating leakage of fibrinogen from the circulation into the alveolar compartment, where it is converted into fibrin by thrombin. This hypothesis is supported by reduced hyperoxia-induced pulmonary vascular leakage in experimental BPD after rolipram or piclamilast treatment (this study) or treatment with the methylxanthine derivative pentoxifylline (5), and attenuated vascular leakage by PDE4 inhibitors in adult lungs exposed to platelet-activating factor, LPS or LPS plus zymosan (19, 29, 39). A role for PDE4 inhibition in vascular leakage is supported by the observation that endothelial vascular leakage is inhibited by cAMP-induced cell junction tightening, which can, at least in part, be
explained by activation of exchange protein directly activated by cAMP (Epac)-1 by cAMP, studied in human umbilical cord endothelial cells (40).

PDE4 inhibition was superior over pentoxifylline in treating experimental BPD, as demonstrated by a more pronounced effect on survival and, in contrast with pentoxifylline, reduced septum thickness, accumulation of monocytes and macrophages and expression of proinflammatory cytokines and chemokines (5). We used a relatively low dosage of rolipram (0.25 mg/kg/day) due to its adverse effects on food intake. Rolipram binds with high affinity to the HARBS (high affinity rolipram binding site) conformation of PDE4 that may functionally be relevant in the central nervous system and parietal glands and account for adverse effects, such as nausea, vomiting and gastric acid secretion (41, 42). Rolipram has lower affinity to the LARBS (low affinity rolipram binding site) conformer of PDE4 that correlates with some anti-inflammatory effects. Higher rolipram dosages up to 5 mg/kg/day resulted in starvation and death of the pups due to cannibalism of the foster mother within the first week of treatment, demonstrating its narrow therapeutic window. To overcome this pitfall we also used a second generation PDE4 inhibitor that does not discriminate between high and low affinity rolipram binding sites on PDE4 and may inhibit PDE4 in immunocompetent cells more efficiently and with less severe side-effects (13, 14). To compare both PDE4 inhibitors as therapy for experimental BPD, we selected the piclamilast dosage (5 mg/kg/day) that had a similar effect on growth retardation as the most effective rolipram dosage (0.25 mg/kg/day, i.e. a more than 10-fold lower molar concentration than piclamilast). Piclamilast outperformed rolipram as a PDE4 inhibitor with a more pronounced effect on fibrin deposition, vascular alveolar leakage and differential mRNA expression in experimental BPD. The large difference in molar concentration of both PDE4 inhibitors can be attributed to differences in pharmacokinetics between both
compounds and, at least in part, by the lower affinity of rolipram to LARBS. This allows the use of a higher concentration of piclamilast with a similar side-effect, but a more pronounced beneficial effect in experimental BPD, compared to rolipram treatment.

In neonatal intensive care methylxanthines, including theophylline, aminophylline and caffeine are widely used for the treatment of apnea of prematurity and weaning from the ventilator, because they increase respiratory drive and, as a result, increases oxygen consumption (3, 4, 43-45). Beneficial effects are related to non-specific inhibition of adenosine A1 and A2a receptors, stimulation of the respiratory center and improved function of the respiratory muscles (46-48). Theophylline treatment in premature infants improved lung function by stimulating spontaneous breathing, reducing the number of apneas and increasing tidal volumes (49). Potential harmful effects of neonatal methylxanthine therapy on growth, neurological development and childhood behaviour were investigated in the caffeine for apnea of prematurity (CAP) trial. Despite a diminished weight gain for 3 weeks, caffeine therapy improved survival without neurodevelopmental disability at 18 and 21 months (50). Immunomodulatory effects of methylxanthines are mediated via non-specific inhibition of PDEs, leading to increased intracellular cAMP or cGMP levels (12-14). The selective PDE5 inhibitor sildenafil has been used in newborns with severe persistent pulmonary hypertension (51, 52), a late complication of BPD. Sildenafil improves alveolar growth and pulmonary hypertension in rat pups exposed to oxidative stress (53). PDE4 inhibitors are under clinical investigation as a novel therapeutic strategy for adult inflammatory lung diseases, like asthma and COPD. The selective PDE4 inhibitors cilomilast and roflumilast have been evaluated in clinical trials (phase III) for COPD, but these drugs have not been approved yet (13, 14, 24, 54). PDE4 inhibitors have not yet been evaluated as a therapeutic strategy for neonatal inflammatory lung diseases such as BPD. The
beneficial effects of rolipram and piclamilast on lung inflammation, extravascular fibrin deposition and survival in neonatal rats with hyperoxic lung injury emphasize the potential of PDE4 inhibitors as treatment for BPD in premature infants.
Acknowledgements

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Legends

Figure 1

Western blot analysis of fibrin deposition in lung homogenates of rat pups exposed to room air (RA), oxygen (O$_2$) and O$_2$ in combination with 2.5 mg/kg/day of piclamilast (Pic$_{2.5}$) or 5.0 mg/kg/day of piclamilast (Pic$_{5.0}$) for 10 days (panel A). Panel B shows the quantification of fibrin deposition in lung homogenates on day 10. Experimental groups include room air controls (RA, white bar), age-matched oxygen-exposed controls (O$_2$, black bar) and rat pups treated with the PDE4 inhibitors rolipram (125 µg/kg/day: striped bar; 250 µg/kg/day: grey bar) or piclamilast (2.5 mg/kg/day: striped bar; 5.0 mg/kg/day: grey bar). Data are expressed relative to the oxygen-exposed control as mean ± SEM of at least 10 pups per experimental group. The 100% value is 357.8 and 258.4 ng fibrin/mg tissue for the rolipram and the piclamilast group, respectively. The number above the PDE4 inhibitor bars indicates the fold-difference in fibrin deposition between age-matched oxygen-exposed controls and PDE4 inhibitor-treated pups. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus age-matched oxygen-exposed controls. In panels C-E paraffin lung sections are depicted at a 200-fold magnification of a rat pup exposed to room air (panel C), after exposure to 100% oxygen (panel D) and after treatment of oxygen-exposed pups with piclamilast (5.0 mg/kg/day) for 10 days (panel E). Sections were stained with the monoclonal anti-human fibrin antibody 59D8, which specifically detects rat β-fibrin. Arrows in panel D indicate fibrin deposits in septa and in the alveolar lumen, associated with the alveolar inner membrane of the lung.
Figure 2

Growth in rat pups treated with the PDE4 inhibitors rolipram (125 µg/kg/day: ▼ and 250 µg/kg/day: ●; panel A) or piclamilast (5.0 mg/kg/day: ●; panel C) and age-matched oxygen-exposed controls (∆) during the first 14 days after birth. Data are expressed as mean ± SEM. Kaplan-Meier survival curves of rat pups treated with rolipram (125 µg/kg/day: ▼ and 250 µg/kg/day: ●; panel B) or piclamilast (5.0 mg/kg/day: ●; panel D) and age-matched oxygen-exposed controls (∆) during the first 23 days after birth. Data are expressed as percentage ± SEM of pups surviving at the observed time point. At least 12 pups per experimental group were studied. *p < 0.05 and ***p < 0.001 for PDE4 inhibitor-treated pups versus age-matched oxygen-exposed controls. ▲▲p < 0.01 versus pups treated with 125 µg/kg/day of rolipram.
Figure 3

Paraffin lung sections stained with monoclonal ED-1 (panels A-D) of room-air (RA, panel A) and oxygen-exposed controls (panel B), and age-matched pups treated with the PDE4 inhibitors rolipram (250 µg/kg/day; panel C) or piclamilast (5.0 mg/kg/day; panel D) under hyperoxia at 10 days of age. Pictures were taken at a 200x magnification. Panel E demonstrates the quantification of ED-1 positive monocytes and macrophages and panel F the mean linear intercept on paraffin sections in room air-exposed littermates (white bars), oxygen-exposed control pups (black bars) and rat pups treated with a PDE4 inhibitor: rolipram (250 µg/kg/day: grey bar) or piclamilast (5.0 mg/kg/day; grey bar). Data are expressed relative to the oxygen-exposed control as mean ±
SEM of at least 6 different rat pups per experimental group. The 100% value is 1349 and 1528 ED-1 positive cells per mm$^2$ (panel E) for the rolipram and the piclamilast group, respectively, and the 100% value is 54.6 and 65.9 µm (panel F) for the rolipram and the piclamilast group, respectively. The number above the PDE4 inhibitor bars indicates the fold-difference in ED-1 positive cells between age-matched oxygen-exposed controls and PDE4 inhibitor-treated pups. Note the presence of large numbers of leukocytes, including macrophages and neutrophils in thickened septa and in the enlarged alveolar lumen in panel B in oxygen-exposed controls, and low numbers of pulmonary inflammatory cells after PDE4 inhibitor treatment (panels C and D). $a$ = alveolus. $^*p < 0.05$ and $^{** *}p < 0.001$ versus oxygen-exposed controls. $^{\Delta}p < 0.05$ versus rat pups in room air.

Figure 3
Figure 4

Total protein concentration in bronchoalveolar lavage fluid (BALF) of room air-exposed controls (white bars), oxygen-exposed control pups (black bars) and rat pups treated with the PDE4 inhibitors rolipram (250 µg/kg/day: grey bar) or piclamilast (5.0 mg/kg/day: grey bar) on day 10. Data are expressed relative to the oxygen-exposed control as mean ± SEM of at least 8 different rat pups per experimental group. The 100% value is 233 and 1223 µg/ml for the rolipram and the piclamilast group, respectively. The number above the PDE4 inhibitor bars indicates the fold-difference in protein concentration between oxygen-exposed controls and PDE4 inhibitor-treated pups. *p < 0.05 and **p < 0.01 versus oxygen-exposed controls.
Figure 5

Relative mRNA expression, determined with RT-PCR, of genes related to inflammation (IL-6 [panel A], CINC-1 [panel B], MCP-1 [panel C] and amphiregulin [panel D]) in room air-exposed controls (RA, white bars), age-matched oxygen-exposed controls (O₂, black bars) and rat pups treated with the PDE4 inhibitors rolipram (125 µg/kg/day: striped bar; 250 µg/kg/day: grey bar) or piclamilast (2.5 mg/kg/day: striped bar; 5.0 mg/kg/day: grey bar) on day 10. Data are expressed as mean ± SEM of 10 rat pups. The number above the PDE4 inhibitor bars indicates the fold-difference in relative expression between oxygen-exposed controls and PDE4 inhibitor-treated pups. *p < 0.05 and ***p < 0.001 versus oxygen-exposed controls. ΔΔp < 0.01 and ΔΔΔp < 0.001 versus room air-exposed rat pups. Ωp < 0.05 and ΩΩp < 0.01 versus piclamilast-treated (5.0 mg/kg/day) rat pups.
Relative mRNA expression, determined with RT-PCR, of genes related to coagulation (TF [panel A] and EPCR [panel B]) in room air-exposed controls (RA, white bars), age-matched oxygen-exposed controls (O2, black bars) and rat pups treated with the PDE4 inhibitors: rolipram (125 µg/kg/day: striped bar; 250 µg/kg/day: grey bar) or piclamilast (2.5 mg/kg/day: striped bar; 5.0 mg/kg/day: grey bar) on day 10. Data are expressed as mean ± SEM of 10 rat pups. The number above the PDE4 inhibitor bars indicates the fold-difference in relative expression between oxygen-exposed controls and PDE4 inhibitor-treated pups. ***p < 0.001 versus oxygen-
exposed controls. $^\Delta p < 0.05$ and $^{\Delta \Delta \Delta} p < 0.001$ versus own room air-exposed rat pups. $^{\Omega \Omega \Omega} p < 0.01$ versus piclamilast-treated (5.0 mg/kg/day) rat pups.

Figure 7

Relative mRNA expression, determined with RT-PCR, of genes related to fibrinolysis (PAI-1 [panel A], tPA [panel B], uPA [panel C] and uPAR [panel D]) in room air-exposed controls (RA, white bars), oxygen-exposed controls (O$_2$, black bars) and rat pups treated with the PDE4 inhibitors: rolipram (125 µg/kg/day: striped bar; 250 µg/kg/day: grey bar) or piclamilast (2.5 mg/kg/day: striped bar; 5.0 mg/kg/day: grey bar) on day 10. Data are expressed as mean ± SEM of 10 rat pups per experimental group. The number above the PDE4 inhibitor bars indicates the
fold-difference in relative expression between oxygen-exposed controls and PDE4 inhibitor-treated pups. *$p < 0.05$ and $***p < 0.001$ versus oxygen-exposed controls. $\Delta \Delta p < 0.01$ and $\Delta \Delta \Delta p < 0.001$ versus room air-exposed rat pups. $\Omega p < 0.05$ versus piclamilast-treated (5.0 mg/kg/day) rat pups.

Figure 8

Relative mRNA expression, determined with RT-PCR, of genes related to alveolar growth (VEGFR2 [panel A] and FGFR4 [panel B]) in room air-exposed controls (RA, white bars), oxygen-exposed controls (O2, black bars) and rat pups treated with the PDE4 inhibitors: rolipram (125 µg/kg/day: striped bar; 250 µg/kg/day: grey bar) or piclamilast (2.5 mg/kg/day: striped bar; 5.0 mg/kg/day: grey bar) on day 10. Data are expressed as mean ± SEM of 10 rat pups. The number above the PDE4 inhibitor bars indicates the fold-difference in relative expression between age-matched oxygen-exposed controls and PDE4 inhibitor-treated pups. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus oxygen-exposed controls. $\Delta \Delta \Delta p < 0.001$ versus room air-exposed rat pups.
Alveolar enlargement

Figure 8
List of abbreviations

ARDS  acute respiratory distress syndrome
BALF  bronchoalveolar lavage fluid
BPD  bronchopulmonary dysplasia
CINC-1 chemokine-induced neutrophilic chemoattractant-1
COPD  chronic obstructive pulmonary disease
EPCR  endothelial protein c receptor
FGFR4 fibroblast growth factor receptor-4
IL  interleukin
MCP-1 monocyte chemoattractant protein-1
PAI-1 plasminogen activator inhibitor-1
RA  room air
RT-PCR reverse transcriptase polymerase chain reaction
TF  tissue factor
tPA  tissue-type plasminogen activator
uPA  urokinase-type plasminogen activator
uPAR urokinase-type plasminogen activator receptor
VEGFR2 vascular endothelial growth factor (VEGF) receptor-2
References


42. Barnette MS, Grous M, Cieslinski LB, Burman M, Christensen SB, Torphy TJ. Inhibitors of Phosphodiesterase IV (PDE IV) increase acid secretion in rabbit isolated gastric glands: correlation between function and interaction with high-affinity rolipram binding site. *J Pharmacol Exper Ther* 1995;273:1396-1402.


Table 1. Sequences of Oligonucleotides Used as Forward and Reverse Primers for Real-Time RT-PCR.

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<thead>
<tr>
<th>Gene Product</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Amphiregulin</td>
<td>5'-TTTCGGCTGGCGCTCTCA-3'</td>
<td>5'-TTCCAACCCAGCTGCATAATG-3'</td>
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<td>CINC-1</td>
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<td>5'-CAGAAGCCAGCGTTCACCA-3'</td>
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<td>EPCR</td>
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<td>5'-AGCAGCTAACAGTCAGAGGAAGAC-3'</td>
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<td>IL-6</td>
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<td>5'-TGTTAAACAGATCGTGAGGTACAG-3'</td>
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<tr>
<td>uPA</td>
<td>5'-ACAGCAGTCCAGGACCATAACA-3'</td>
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