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Detrimental Effects of an Inhaled Phosphodiesterase-4 Inhibitor on Lung Inflammation in Ventilated Preterm Lambs Exposed to Chorioamnionitis Are Dose Dependent

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Abstract

Background: Treatment of bronchopulmonary dysplasia in preterm infants is challenging due to its multifactorial origin. In rodent models of neonatal lung injury, selective inhibition of phosphodiesterase 4 (PDE4) has been shown to exert anti-inflammatory properties in the lung. We hypothesized that GSK256066, a highly selective, inhalable PDE4 inhibitor, would have beneficial effects on lung injury and inflammation in a triple hit lamb model of Ureaplasma parvum (UP)-induced chorioamnionitis, prematurity, and mechanical ventilation.
Methods: Twenty-one preterm lambs were surgically delivered preterm at 129 days after 7 days intrauterine exposure to UP. Sixteen animals were subsequently ventilated for 24 hours and received endotracheal surfactant and intravenous caffeine citrate. Ten animals were randomized to receive twice a high (10 μg/kg) or low dose (1 μg/kg) of nebulized PDE4 inhibitor.
Results: Nebulization of high, but not low, doses of PDE4 inhibitor led to a significant decrease in pulmonary PDE activity, and was associated with lung injury and vasculitis, influx of neutrophils, and increased proinflammatory cytokine messenger RNA levels.
Conclusion: Contrary to our hypothesis, we found in our model a dose-dependent proinflammatory effect of an inhaled highly selective PDE4 inhibitor in the lung. Our findings indicate the narrow therapeutic range of inhaled PDE4 inhibitors in the preterm population.

Keywords: bronchopulmonary dysplasia, lung injury, mechanical ventilation of neonates, PDE4 inhibitors, prematurity

Introduction

Bronchopulmonary dysplasia (BPD) continues to be the major morbidity in infants born prematurely.[1,2] Among others, BPD has been linked to an early inflammatory reaction in the developing lung associated with chorioamnionitis.[1,3] Exposure to chorioamnionitis has also been shown to increase the risk of BPD development derived from mechanical ventilation,[4] another yet important factor originally linked to the pathophysiology of BPD.[1] In consequence of the multifactorial origin of BPD, targeted prevention and therapies are scarce.[5] Currently, anti-inflammatory therapies are used in BPD prevention and therapy, especially postnatal glucocorticosteroids.[6] However, concerns about adverse effects such as impaired neurodevelopmental outcome after systemic use of glucocorticosteroids[7] have raised the demand to develop new anti-inflammatory therapies.[5]

Potential candidates for targeting neonatal lung inflammation are phosphodiesterase (PDE) inhibitors, such as

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methylxanthines and their derivates.[8] Caffeine, a methylxanthine widely used in the treatment of apnea in preterm infants, has been shown to decrease the incidence of BPD.[9] Although the mechanism is still discussed, caffeine's property as a weak nonspecific PDE inhibitor might contribute to its beneficial effect on BPD.[10] Another methylxanthine, pentoxifylline (PTXF), has been shown to exert anti-inflammatory effect on neonatal monocytes *in vitro*.[11] In a clinical pilot study, PTXF decreased the incidence of BPD when nebulized to very low birth weight preterm infants.[12] However, this beneficial effect could not be reproduced in a clinical study on extremely preterm infants with a high risk of developing BPD.[13] A more specific inhibition of PDE isoenzymes might, therefore, increase the therapeutic benefit.

Of the reported isoforms, PDE4 is the main enzyme in lung and inflammatory cells, which reduces cleavage of cyclic AMP (cAMP).[14] Therefore, in recent years, PDE4 inhibitors have been developed to target different adult lung diseases such as asthma and chronic obstructive pulmonary disease (COPD).[15] In the context of BPD, selective inhibition of PDE4 has been tested in different rodent models. In these studies, anti-inflammatory effects of PDE inhibition were identified as a possible mechanism to protect the lung from hyperoxia-induced BPD phenotype[16–19] and from lipopolysaccharide (LPS)-induced lung inflammation.[20–23]

We, therefore, hypothesized that a new highly selective PDE4 inhibitor optimized for inhaled delivery (GSK256066)[21,22] would exert anti-inflammatory properties in the lungs of preterm lambs exposed to chorioamnionitis and subsequent mechanical ventilation.

Materials and Methods

Preparation of iPDE4

A stock solution of GSK256066 (Selleckchem, Munich, Germany) was made according to the manufacturer's guidelines by dissolving 2.5 mg GSK256066 in 1 mL of 20% dimethyl sulfoxide (DMSO). Two working solutions were prepared with a final concentration of 50 and 5 μg/mL GSK 256066, both in 2% DMSO, and frozen at −20°C. Immediately before administration, the working solution was thawed, and an amount of 0.2 mL/kg body weight was mixed with the same amount of 0.9% NaCl, before filling a vibrating membrane nebulizer (eFlow® Neonatal Nebulizer System; PARI Pharma, Munich, Germany). The lower dose of 1 μg/kg was based on ED_{50} value of 1.1 μg/kg identified in a rat model of LPS-induced lung inflammation[21] and resembles the adult dose of 87.5 μg used in clinical trials on COPD.[24] The higher dose of 10 μg/kg was based on previous rodent studies describing anti-inflammatory effects after LPS inhalation.[22]

Animal study

The study design and the experimental protocol were in line with the institutional guidelines for animal experiments and were approved by the institutional Animal Ethics Research Committee of Maastricht University and the Dutch Central Animal Research Commission (CCD).

Seven days before delivery, 21 date-mated ewes underwent ultrasound-guided intra-amniotic injection of Ureaplasma parvum (UP) (strain HPA 5), 5×10^5 color changing units. One day before cesarean section, ewes were injected intramuscularly with β-methasone (12 mg, Celestone®; Schering-Plough, North Ryde, NSW, Australia). Before delivery, lambs were randomly assigned to four different treatment groups: nonventilated controls that were sacrificed immediately (NOVENT), animals ventilated for 24 hours without iPDE4 treatment (Control), and two groups of ventilated animals that received 1 μg/kg GSK256066 (iPDE1) or 10 μg/kg GSK256066 (iPDE10), respectively, at 30 minutes and 12 hours postnatal age (Fig. 1).

Lambs were surgically delivered at a gestational age of 129 days (term ~150 days), equipped with umbilical artery and vein catheters and intubated orally before clamping the cord and weighing.[25] Animals in the ventilation groups were transferred to an infant radiator bed (IW930 Series Cosy-Cot™ Infant Warmer; Fisher & Paykel, Auckland, New Zealand) and connected to an infant ventilator (Fabian HFO®; Acutronic, Hirzel, Switzerland) with the following initial settings: SIMV, PIP 30 cmH_2O, PEEP 8 cmH_2O, ventilation rate 50/min, and FiO_2 0.40. Subsequently, animals received an endotracheal dose of 200 mg/kg body weight Poractant alpha (Curosurf®; Chiesi Pharmaceuticals, Parma, Italy) and a single loading dose of caffeine citrate i.v. (20 mg/kg, Peyona®; Chiesi Pharmaceuticals). The nebulizer was prepared as already described and placed between the tube and the connection to the ventilator circuit.[26] Ventilation was adjusted to blood gas analysis to maintain pO_2 between 60 and 90 mmHg and pCO_2 between 45 and 70 mmHg (iStat device; Point of Care, Inc., Abbott Park, IL). During the experimental period of 24 hours, lambs were continuously sedated with midazolam (Actavis, Hafnarfjordur, Iceland) and ketamine (Alfasan B.V., Woerden, The Netherlands), and parenterally fed with a 1:1 mixture of glucose 20% and Ringer's solution (B. Braun Medical B.V., Oss, The Netherlands).

Necropsy

At the end of the experiment, lambs were euthanized by an intravenous injection of 10 mL pentobarbital. The thorax was opened and the lungs were removed, divided into lobes, and weighed. The right upper lobe (RUL) was inflation fixed in 10% buffered paraformaldehyde for 24 hours. Lung tissue from the right middle lobe (RML) was snap frozen. Paraffin-embedded RUL sections (4 μm) were stained with

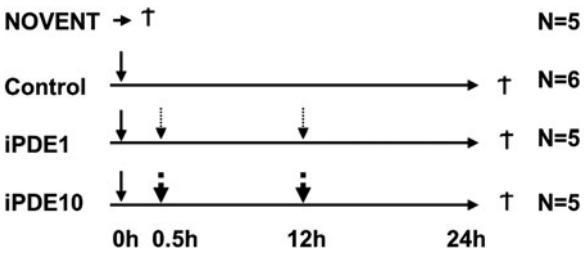

FIG. 1. Experimental groups. After initiation of ventilation, animals received one dose of Curosurf® 200 mg/kg intratracheally and 20 mg/kg caffeine intravenously (arrow). Animals in the treatment groups received 1 μg/kg (thin dotted arrows) or 10 μg/kg PDE4 inhibitor (thick dotted arrows) through nebulization at 0.5 and 12 hours. PDE, phosphodiesterase.

hematoxylin and eosin before semiquantitative scoring of lung injury, based on the composite score published by Hillman et al.[27]

Immunohistochemistry

Paraffin-embedded RUL lung sections (4 μm) were stained for CD3 (DAKO A0452; Dakocytomation, Glostrup, Denmark) and myeloperoxidase (MPO) (DAKO A039829; Dakocytomation). In brief, the sections were deparaffinized in an ethanol series. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in 1 × phosphate-buffered saline (PBS; pH 7.4). Antigen retrieval was performed by heating the sections in heated citrate buffer (10 mM, pH 6.0) for 10 minutes. To block nonspecific binding, the slides were incubated with 5% bovine serum albumin in PBS (for CD3) or 20% normal goat serum in PBS (for MPO). For CD3, sections were incubated overnight at 4°C with the diluted primary antibody (1:200, DAKO A0452; Dakocytomation). After incubation with a swine-antirabbit biotin-labeled secondary antibody (DAKO E0353; Dakocytomation), immunostaining was enhanced with Vectastain ABC Peroxidase Elite Kit (PK-6200; Vector Laboratories, Burlingame, CA) and stained with nickel sulfate–diaminobenzidine. Subsequently, the sections were rinsed in Tris/saline and incubated with Tris/cobalt. Counterstaining was performed with 0.1% Nuclear Fast Red. For MPO, sections were incubated for 1 hour with the 1:500 diluted primary antibody (MPO, Dako A0398). After incubation with the 1:200 diluted secondary antibody (Peroxidase Goat Anti-Rabbit IgG, 111-035-045; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), slides were incubated with 0.02% 3-amino-9-ethylcarbazole (Sigma A5754) dissolved in sodium acetate $C_2H_3NaO_2$ (0.05M, pH4.9) and a total of 0.01% H_2O_2 (Sigma H1009). After washing, background staining was performed with hematoxylin. For analysis, slides were scanned (Ventana iScan HT; Roche Diagnostics, Basel, Switzerland) and pictures were taken at 200 × magnification with the Ventana Imageviewer (Roche Diagnostics). MPO- and CD3-positive cells were counted in five representative high-power fields by a blinded observer and averaged per animal.

PDE activity

PDE activity was calculated from cAMP concentration as described previously.[23] Frozen lung tissue was homogenized in a buffer consisting of 30 mM HEPES and 0.1% Triton X-100 (a total volume of 4 μL/mg lung). After 10 minutes centrifugation at 13,000 × g, 10 μL lung homogenate was mixed with 190 μL PDE assay buffer (137 mM NaCl, 2.7 mM KCl, 8.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 1 mM $CaCl_2$, and 1 mM $MgCl_2$), and adding 1 μM cAMP started the reaction (incubated at 10 minutes at 37°C) and reaction stopped by boiling for 3 minutes. After centrifugation at 12,000 × g for 30 minutes, the cAMP concentrations in the supernatants were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Enzo Life Sciences, Farmingdale, NY). PDE activity was calculated reciprocally in all but one animal wherein cAMP was outside the threshold of detection.

RNA extraction and real-time PCR

Total RNA was isolated from the RML using NucleoSpin® RNA Kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's protocol. For quantification of total RNA, a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) was used as recommended by the manufacturer. Total RNA was eluted in 60 μL nuclease-free H_2O (Sigma-Aldrich) and stored at −80°C until reverse transcription. For real-time PCR (RT-PCR), 1 μg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was diluted 1:10 with deionized nuclease-free H_2O (Sigma-Aldrich) and stored at −20°C until required for further analysis.

Quantitative RT-PCR

For quantitative detection of messenger RNA (mRNA), 10 μL of diluted first-strand cDNA was analyzed in duplicates of 25 μL reactions using 12.5 μL iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 μL deionized H_2O, and 1 μL of a 10 μM solution of forward and reverse primers (Sigma-Aldrich). Levels of mRNA were measured for inflammatory cytokines interleukin (IL)-1, IL-6, and IL-8, for tumor necrosis factor (TNF)-α, and for tissue inhibitor of metalloproteinase 1 (TIMP1). TIMP1 has been described as a predictive biomarker for mesenteric vasculopathy induced by PDE4 inhibition.[28] Primers were ovIL1Bfwd 5′-CCTGTCATCTTC GAAACATCC-3′, ovIL1Brev 5′-GCAGAACACCACTTC TCGG-3′, ovIL-6fwd 5′-CTCTCATTAAGCACATCGT-3′, ovIL-6rev 5′-GATCAAGCAAATCGCCTG-3′, ovIL-8fwd 5′-AAACACATTCCACACCTTTCC-3′, ovIL-8rev 5′-GG ATCTTGCTTCTCAGCTCTC-3′, ovTNFafwd 5′-ACACTC AGGTCATCTTCTC-3′, ovTNFarev 5′-GGTTGTCTTTCA GCTCCA-3′, ovTIMP1fwd 5′-ACTCCGAAGTCGTCAT CAG-3′, ovTIMP1rev 5′-GAAGTATCCGCAGACGCTC-3′, ovACTBfwd 5′-ATCTGTCGTCAGCAGGTC-3′, ovACTBrev 5′-CCAACGGTACTGAGAGGA-3′. PCRs were performed on an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific) using a two-step PCR protocol after an initial denaturation at 95°C for 10 minutes with 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A melt curve analysis was performed at the end of every run to verify single PCR products. Levels of mRNAs were normalized to those of β-actin. Mean fold changes in mRNA expression were calculated by the $\Delta\Delta C_T$ method by Livak and Schmittgen.[29]

Cytokines ELISA in tracheal aspirate

During ventilation, tracheal aspirates (TAS) were obtained at 0, 12, and 24 hours by giving 4 mL of 0.9% NaCl into the endotracheal tube, followed by five consecutive inflations and subsequent endotracheal suction. Levels of the proinflammatory cytokines IL-6 and IL-8 were measured in TAS using ovine-specific sandwich ELISAs. A 96-well plate was coated with a monoclonal mouse anti-IL-6 (Millipore cat. no. MAB1004, working concentration 1:200) or IL-8 (Millipore cat. no. MAB1044, working concentration 1:200) and incubated overnight at 4°C. The standard curve

Table 1. Animal Characteristics

	NOVENT	*Control*	*iPDE1*	*iPDE10*
N	5	6	5	5
Male:female	1:4	2:4	2:3	1:4
Birth weight (kg)	2.8 ± 0.2	3.1 ± 0.2	2.8 ± 0.2	3.4 ± 0.3
Relative weight loss during experiment	n.a.	−0.5%	−2.4%	−4.8%

n.a., not applicable; PDE, phosphodiesterase.

and TAS samples were diluted in PBS +0.1% BSA +0.05% tween in 1:1 or 1:100, respectively, for IL-6 and IL-8. Incubation for 1 hour with the detection antibody rabbit-antiovine IL-6 (Millipore cat. no. AB1839, working concentration 1:500) or IL-8 (Millipore cat. no. AB1040, working concentration 1:500) was followed by incubation with a horseradish peroxidase (HRP)-labeled antibody (Goat-anti-HRB; Jackson ImmunoResearch Labs cat. no. 111-035-045, working concentration 1:500, Streptavidine-HRP ref P105209 1:40). Subsequent incubation with 3,3′5,5′-tetramethylbenzidine substrate solution was done for 10 (IL-6) or 2.5 (IL-8) minutes, and the reaction was stopped by addition of H_2SO_4. Optical density was measured at 450 nm in a Thermo Electron Type 1500 Multiskan Spectrum Microplate Reader (Thermo Fisher Scientific), and concentrations were expressed relative to a standard curve of recombinant ovine IL-6 or IL-8 (ImmunoChemistry Technologies, Bloomington, MN).

Statistics

Data are expressed as mean and standard error of means, and statistical analysis was performed using one-way analysis of variance with Bonferroni *post hoc* testing with IBM® SPSS version 20. Graphs were drawn with GraphPad Prism® v5.0. Significance was accepted at $p < 0.05$.

Results

Baseline characteristics

Animals in different groups did not differ significantly in gender, birth weight, and relative weight loss during the experiment (Table 1). Blood gas analysis during ventilation showed stable results during the experimental period, with a mean pCO_2 slightly above the target range at 24 hours in the iPDE1 group (Fig. 2). One animal died at 12 hours due to tension pneumothorax.

PDE activity

At sacrifice, PDE activity was highest in ventilated control animals. In the high-dose group, but not in the low-dose group, PDE activity was significantly decreased by ~80% compared with unventilated and ventilated controls 12 hours after the second dose (Fig. 3).

MPO and CD3

The number of MPO positive cells per high power field indicating neutrophils was low in unventilated and ventilated controls (Fig. 4A), and significantly increased in the iPDE10, but not in the iPDE1 group. CD3 positive cells indicating lymphocytes (Fig. 4B) were found in the lungs of all animals irrespective of ventilation or PDE4 inhibitor treatment.

Cytokines RNA

Levels of proinflammatory cytokines' RNA for IL-1β, IL-6, IL-8, and of TNF-α were lowest in unventilated controls. Higher levels were found in all ventilated groups, however, only animals receiving a high dose of PDE inhibitor showed a significant increase of IL-1β, IL-8, and TNF-α levels ($p < 0.05$ compared with NOVENT, Control, and iPDE1; Fig. 5A,C,D). Cytokine mRNA levels in the low-dose group were comparable with or lower than levels in ventilated controls, but this effect was not statistically significant when comparing all groups. For TIMP1, increase in mRNA levels in ventilated animals was significant only in the iPDE10 group ($p < 0.05$ compared with NOVENT, $p = 0.054$ vs. Control; Fig. 5F).

Lung injury score

The composite injury score in unventilated control animals was low, and ventilation alone did not lead to a significant increase of the score (Fig. 6A–C). Animals in the iPDE10 group showed a significantly higher score for lung injury compared with unventilated and ventilated control animals, with thickened airway walls and parenchymal

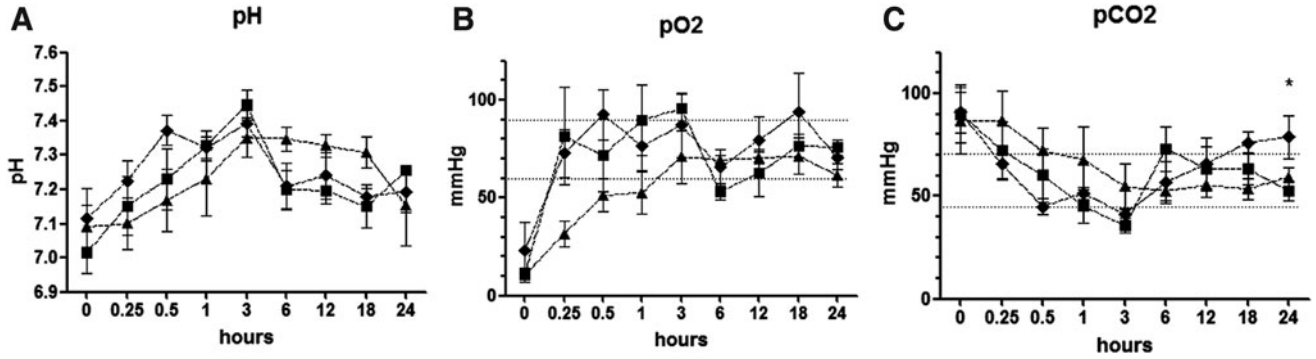

FIG. 2. Blood gas analysis. During ventilation, animals in the Control (square), iPDE1 (diamond), and iPDE10 groups (triangle) showed stable results for **(A)** pH, **(B)** pO_2, and **(C)** pCO_2, with a pCO_2 slightly above the target range (thin dotted lines) at 24 hours in the iPDE1 group (data as mean ± SEM, $*p < 0.05$ vs. Control). SEM, standard error of mean.

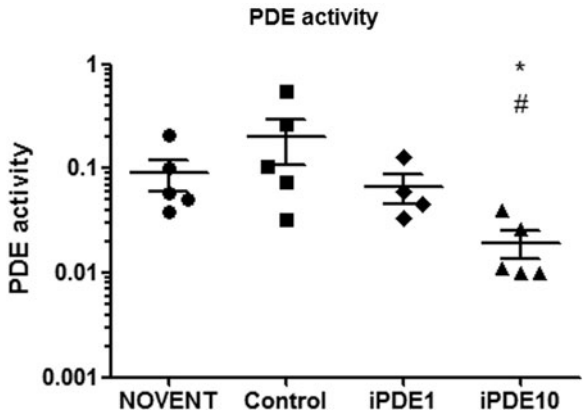

FIG. 3. PDE activity. In lung tissue, PDE activity calculated from cyclic AMP concentration was significantly decreased in the iPDE10 group compared with unventilated and ventilated controls ($p < 0.05$ * vs. NOVENT, # vs. Control).

hemorrhage (Fig. 6E). In this group, infiltration of inflammatory cells could additionally be found in perivascular tissue, indicating vasculitis (Fig. 6F). Lung injury scores in the iPDE1 group were not significantly increased ($p = 0.091$ vs. NOVENT; Fig. 6D).

IL-6 and IL-8 in TAS

IL-6 and IL-8 concentration in TAS increased significantly during the experiment in all ventilated groups (Fig. 7A,B). However, IL-6 in the iPDE1 group showed a distinct different pattern over time from the other groups, with no increase after the first dose and subsequent ventilation for 12 hours ($p < 0.05$ vs. iPDE10).

Discussion

Contrary to our hypothesis, we found proinflammatory effects and increased injury scores in the lungs of preterm lambs nebulized with 10 μg/kg per dose of a selective PDE4 inhibitor. The proinflammatory phenotype in our study was associated with histological signs of vasculitis and an increase in pulmonary mRNA levels of TIMP1, a potential biomarker of intestinal vasculopathy induced by PDE4 inhibitors.[28] Mesenteric and intestinal vasculitis after oral application of PDE4 inhibitors have been described in various animal studies and have been identified as dose-limiting adverse effect.[30,31]

In our study, both PDE inhibition and associated proinflammatory effects were dose dependent. PDE was not significantly inhibited at sacrifice in animals receiving the lower dose of PDE4 inhibitor. Of note, this was 12 hours after the second dose, so we cannot rule out a time-dependent PDE inhibition also after the low dose. However, we found no significant anti-inflammatory effect in the iPDE1 group compared with ventilated controls. In TAS, animals in the iPDE1 group showed a slower increase of IL-6 compared with the iPDE10 group, with a significantly lower concentration after 12 hours, but not after 24 hours. This suggests that inflammatory effects possibly are delayed but not prevented by low doses of the PDE4 inhibitor. Future studies could, therefore, investigate cumulative effects of the low iPDE4 dose during long-term ventilation and with a different dosing interval.

In the iPDE10 group, we observed a strong PDE inhibition 12 hours after the second dose, indicating a total blocking of PDE after administration, possibly resulting from a supratherapeutic dose. This dose was, however, chosen based on previous works in rodents, where doses of 10 μg/kg GSK256066 were administered intratracheally to rats challenged with LPS inhalations. In that study, this dose had anti-inflammatory effects in the lungs and was well tolerated.[22] Our contrary findings might, therefore, arise from differences in the experimental setup, including choice of proinflammatory stimuli, route of administration, and species.

Studies showing anti-inflammatory effects of PDE4 inhibition often used rodent models and *in vitro* testing with LPS as very strong proinflammatory stimulus,[20–23] and found augmented inflammation after exposure to both LPS and PDE4 inhibitor through different routes. Of the mentioned, only one study reported higher neutrophil counts in bronchoalveolar lavage and high levels of lung keratinocyte-derived chemokine (a mouse homologue of the IL-8 family) when PDE4 inhibitor treatment was administered subcutaneously without prior LPS exposure.[20] In our study, we

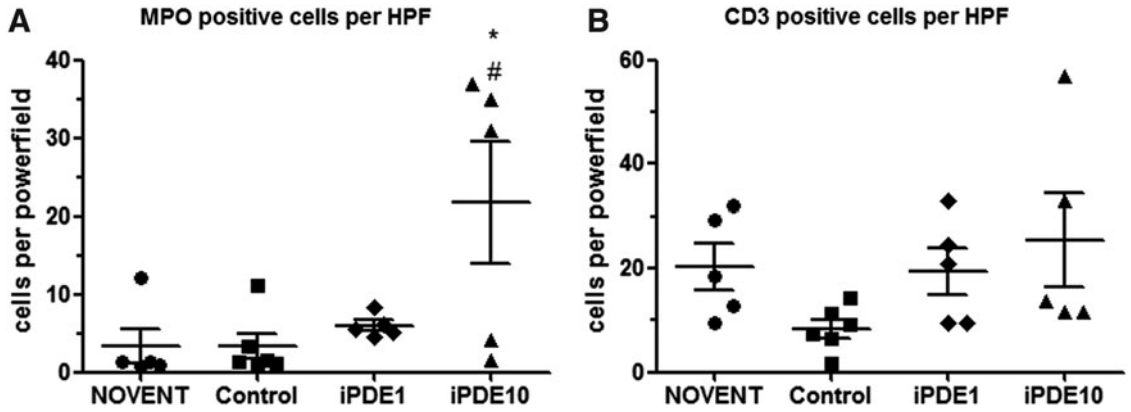

FIG. 4. MPO and CD3. **(A)** MPO positive cells indicating neutrophils but not **(B)** CD3 positive cells indicating lymphocytes were significantly increased in the iPDE10 group compared with NOVENT and control animals ($p < 0.05$ * vs. NOVENT, # vs. Control). HPF, high powerfield; MPO, myeloperoxidase.

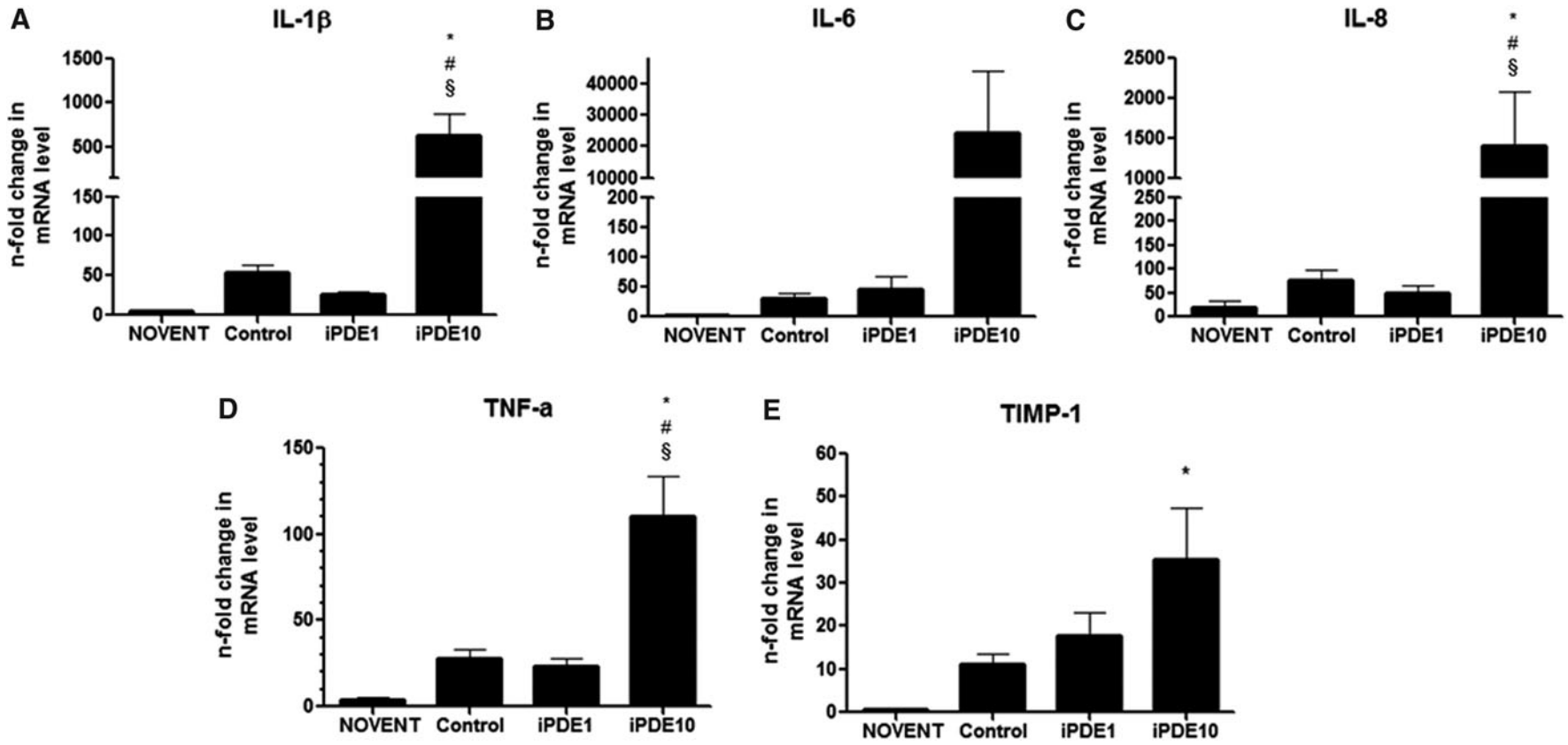

FIG. 5. Cytokines mRNA. Proinflammatory cytokines mRNA levels of IL-1β, IL-6, IL-8, and TNF-α increased in ventilated animals and were significantly higher for IL-1β, IL-8, and TNF-α in the iPDE10 group ($p < 0.05$ * vs. NOVENT, $^{\#}$ vs. Control, § vs. iPDE1) **(A, B, C, D)**. A comparable pattern of increase was shown for TIMP1 **(E)**. IL, interleukin; mRNA, messenger RNA; TIMP1, tissue inhibitor of metalloproteinase 1; TNF, tumor necrosis factor.

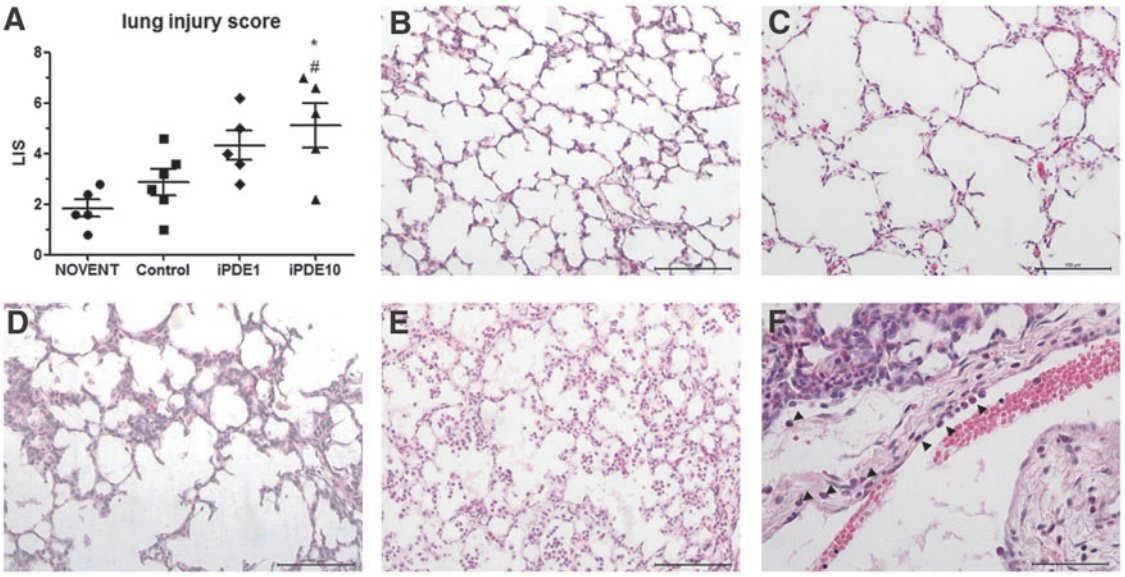

FIG. 6. LIS. Semiquantitative LIS was significantly higher for iPDE10 group animals compared with nonventilated and ventilated control animals ($p < 0.05$ * vs. NOVENT, # vs. Control) **(A)**. Representative lung slides of **(B)** NOVENT, **(C)** Control, **(D)** iPDE1, and **(E)** iPDE10 group. In the iPDE10 group, neutrophil recruitment into vessel walls indicates vasculitis (arrowheads) **(F)**. LIS, lung injury score.

chose a different yet clinically relevant approach to induce preterm lung injury and inflammation. Intrauterine UP exposure has been shown to induce chorioamnionitis and to generate fetal pulmonary and systemic inflammation.[32,33] Although pulmonary inflammation by UP is described as mild, it has been associated with BPD in several studies.[34] Acute UP infection of fetal baboons increased ventilation-associated lung injury and postnatal lung inflammation.[35] However, our data indicated that the phenotype of a more subacute pulmonary inflammation might react differently on PDE4 inhibition than on acute LPS exposure.

Previous studies also explored different routes of PDE4 inhibitor administration. In the hyperoxia-rodent model of neonatal lung injury and BPD,[16–19] where lung inflammation and injury are induced by ongoing oxidative stress postnatally, anti-inflammatory effects of PDE4 inhibition have been reported after subcutaneous or intraperitoneal application of different PDE4 inhibitors. Systemic PDE4 inhibitor treatment has, however, been associated with adverse treatment effects, for example, reduced weight gain.[16,18] In rats treated orally for 2 weeks with supratherapeutic doses of the PDE4 inhibitor rolipram, histological signs of organ damage were found in the heart, vasculature, stomach, and salivary glands, but interestingly not in the lungs of treated animals.[36] To decrease systemic side effects, we chose for nebulization to target the lung directly, using GSK256066 as PDE4 inhibitor, which is suitable for inhalation.[21] Choosing this approach, we observed inflammatory changes in the lung, indicating that the primary site of PDE4 inhibitor exposure implicates the site of major adverse effects.

Our study is limited by the fact that we used DMSO as a solvent for the PDE4 inhibitor. DMSO has been used in

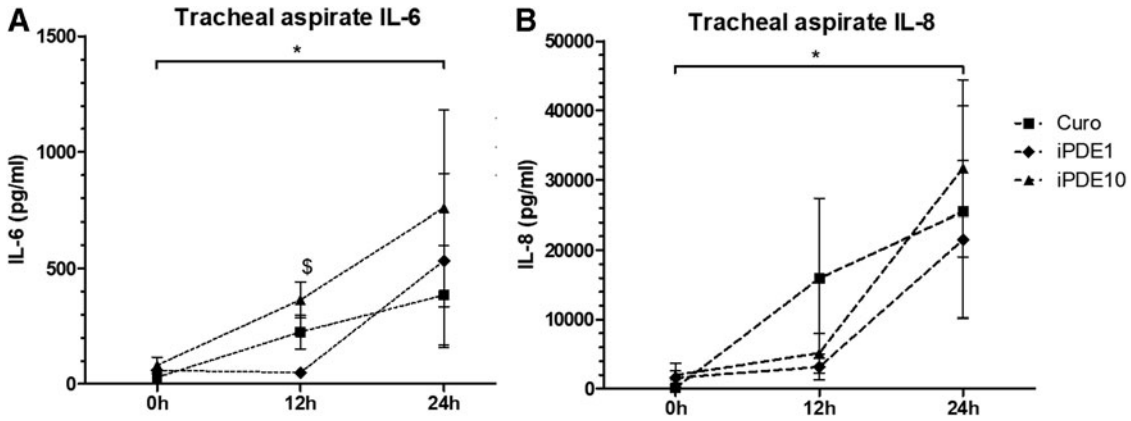

FIG. 7. IL-6 and IL-8 in tracheal aspirates. IL-6 and IL-8 concentration in TAS increased significantly during the experiment in all ventilated groups **(A, B)**. However, animals in the iPDE1 group showed a delayed decrease of IL-6 ($p < 0.05$ $ vs. iPDE10). TAS, tracheal aspirates.

various preclinical models due to its high solubilization capacity and a very low toxicity.[37] For endotracheal use, data is conflicting: in mice, repeated endotracheal exposure against 2% DMSO for 5 days resulted an increase in MPO activity in bronchoalveolar lavage fluid, whereas only small number of neutrophils were recruited to the lung.[37] Endotracheal DMSO further ameliorated neutrophil influx in a hamster model of acute lung injury.[38] Based on these findings and in accordance with good animal experimental practice, we decided that the low risk of significant effects of inhalation with 1% DMSO in our model did not justify a separate control group receiving DMSO only.

Finally, translation of findings to other species and the human situation is difficult. Rats are thought to be very susceptible in terms of PDE4 inhibitor-induced toxicity.[39] To our knowledge, no data from ovine models exist. The preterm lamb model has been chosen due to its similarity to human lung development, and we, therefore, regard our model to be suitable for preclinical testing of pulmonary drug delivery in the context of preterm lung injury and inflammation. However, due to limited number of animals, our model cannot easily be used for more detailed dose-finding studies. It becomes clear that doses from adult humans and rodents cannot be easily translated into preterm settings. Our findings show that dose-dependent toxicity and pulmonary inflammation narrow the therapeutic drug concentration of PDE4 inhibitors dramatically. These findings require thorough investigation of this group of substances before clinical use in preterm infants.

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Author Disclosure Statement

The authors declare they have no competing financial interests.

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