Antenatal Phosphodiesterase 4 Inhibition Restores Postnatal Growth and Pulmonary Development in a Model of Chorioamnionitis in Rabbits


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ABSTRACT

Chorioamnionitis is implicated in the pathophysiology of bronchopulmonary disease, and the associated inflammatory response is responsible for adverse effects on alveolar development. The aim of this work was to analyze the effects of a phosphodiesterase 4 (PDE4)-selective inhibitor, rolipram (a modulator of the inflammatory response), in an experimental model of chorioamnionitis on pulmonary development and on the processes of infection and inflammation. Rabbit mothers were assigned to four groups: 1) saline serum inoculation (controls); 2) *Escherichia coli* intrauterine inoculation (C+); 3) rolipram infusion (R+); and 4) *E. coli* inoculation + rolipram infusion (C+R+). High rates of morbidity and mortality were noticed in mothers and pups (6 of 13 pregnant rabbits in groups with rolipram). Alveolar development, inflammation, and infection were analyzed in pups at day 0 and day 5. At day 0, in the context of chorioamnionitis, rolipram significantly decreased birth weight (p < 0.01) relative to that of controls (p < 0.05). At day 5, weight normalized in group C+R+ but not in group C+ relative to controls (p < 0.001); moreover, alveolar airspace volume was preserved in group C+R+ but not in group C+ (p < 0.05). Interstitial volume decreased in group C+ versus controls (p < 0.05) but was preserved in group C+R+. Specific alveolar area was not significantly modified by rolipram. No significant difference was found concerning bronchoalveolar lavage cellularity, and all blood cultures remained sterile. In this model of impaired alveologenesis, rolipram significantly preserved specific alveolar density. However, PDE4 inhibition induced antenatal fetal demise and growth retardation.

Introduction

Bronchopulmonary dysplasia (BPD) remains a major concern in very premature infants despite recent advances in perinatal care. BPD is characterized by the arrest of secondary septation, previously described as an alveolar number decrease and an alveolar enlargement and is associated with thinned septa and minimal capillary development (Kinsella et al., 2006). Alveolarization occurs postnatally in humans, and premature, in utero exogenous disturbances of immature lungs are thought to induce BPD through inflammatory processes of the airway (Coalson et al., 1999). The main etiologies are the prematurity itself and also perinatal care such as hyperoxia and mechanical ventilation (Kraybill et al., 1989). Relationships between BPD and inflammation were established by Ogden et al. (1983), who found increased inflammatory cell counts in bronchoalveolar lavage (BAL) fluid of neonates exposed to hyperoxia or mechanical ventilation who subsequently developed BPD. Chorioamnionitis and neonatal infections have been implicated in the development of pulmonary defects. In fact, correlation between inflammation, premature delivery, and BPD has been demonstrated in humans (Kallapur et al., 2001) and animals (Kallapur et al.,...
Pregnant and newborn New Zealand White rabbits were used in the study. The RS 218 (O18:K1:H7) strain of *Escherichia coli* K1 was used and has been characterized for virulence factors and invasion genes (Huang et al., 1995). The minimum inhibitory concentration of this strain for ceftriaxone was 0.06 mg/ml. The intrauterine inoculation protocol, as described previously (Gras-Le Guen et al., 2003), was approved by the Animal Care Committee of the University of Nantes experimental therapy department (Nantes, France). A total of 25 female New Zealand White rabbits weighing 3.7 to 4.5 kg (CEGAV, Saint Marc d’Egrenne, France) were obtained 10 days before the end of their usual 31- to 32-day gestation period and housed at the Nantes School of Medicine (Nantes, France) in appropriate cages with a special place for nidation. The 25 female rabbits were randomly assigned to four groups: 1) controls (saline serum inoculation); 2) *E. coli* strain inoculation; 3) rolipram (R6520; Sigma-Aldrich, Lyon, France), according to the experimental group. Posology of rolipram was 0.250 mg/kg in our study. Rolipram was suspended at 7.3 mg/ml in DMSO. The rolipram treatment was started as the antibiotic treatment 6 h after intrauterine inoculation and lasted 4 days until spontaneous delivery. For administration, rolipram was infused with 20 ml of 0.9% saline serum during a 60-min continuous infusion. On day 28 of gestation, the animals were anesthetized with Propofol-Lipuro 1% (B. Braun Pharma, Boulouge-Billancourt, France) and ketamine (25 mg/kg). Laparotomy was then performed via a 2-cm vertical midline incision below the gravid uterus. After the uterus was exposed, a single injection of *E. coli* [1 × 10⁶ colony forming units (CFU)] in 1 ml of 0.9% saline (NaCl solution) or sterile vehicle was performed under visual control into one of the uterine horns facing a fetoplacental unit. Slight aspiration was performed before injection to verify correct intraamniotic positioning of the needle. The incision was then closed in layers, and the animals were returned to their cages.

After spontaneous birth, rabbit pups were suckled until euthanasia. They were exsanguinated at birth (day 0) or on day 5, under deep anesthesia with intraperitoneal thiopental (Pentothal). Blood was obtained by cardiac puncture after animals failed to blink an eyelid or move a leg muscle after local stimulation. Five to 10 rabbit pups/group were used for either bronchoalveolar lavage or morphometric study.

### Assessment of Lung Morphometry

Lungs were gently extracted and fixed with 4% paraformaldehyde through a polyethylene tracheal cannula at a constant pressure of 30 cm H₂O for 10 min. The trachea was then ligated, and the lung was immersed in 4% paraformaldehyde for 24 h. Lung volumes were measured by the displacement method in the fixative solution (Scherle, 1970). Both lungs of each rabbit were paraffin-embedded, and 4-μm frontal sections were cut in the medial part of the lungs and stained with hematoxylin-eosin-saffron. All lung lobes were used. All morphometric evaluations were performed by one observer (L.H.) blinded to group assignment. A microscope (Leitz, Wetzlar, Germany) connected to a television screen by a video camera (Sony, Tokyo, Japan) was used. Volume densities of pulmonary parenchymal structures (alveolar airspace, airways, blood vessels larger than 20 μm in diameter, and interstitial tissues), and alveolar surface density were measured using the point counting and mean linear intercept methods, as described previously (Weibel and Cruz-Orive, 1997). Light microscope fields were quantified at an overall magnification of 400×, using a 42-point, 21-line eyepiece graticule placed on the television screen. Twenty fields (10/lung) were evaluated per animal by a systematic sampling method from a random starting point. To correct for shrinkage associated with fixation and paraffin processing, area values were multiplied by 1.22, a factor calculated during a previous evaluation (Franco et al., 2002). All morphometric data were expressed as relative and absolute values, as described by Burri et al. (1974). Relative values (volume density or surface density) were those obtained directly from morphometric measurements of tissue sections. Absolute values (total volume or surface area per lung) were determined by multiplying the relative values by lung volume.

### Materials and Methods

#### Antenatal Infection

Pregnant and newborn New Zealand White rabbits were used in the study. The RS 218 (O18:K1:H7) strain of *Escherichia coli* K1 was used and has been characterized for virulence factors and invasion genes (Huang et al., 1995). The minimum inhibitory concentration of this strain for ceftriaxone was 0.06 mg/ml. The intrauterine inoculation protocol, as described previously (Gras-Le Guen et al., 2003), was approved by the Animal Care Committee of the University of Nantes experimental therapy department (Nantes, France). A total of 25 female New Zealand White rabbits weighing 3.7 to 4.5 kg (CEGAV, Saint Marc d’Egrenne, France) were obtained 10 days before the end of their usual 31- to 32-day gestation period and housed at the Nantes School of Medicine (Nantes, France) in appropriate cages with a special place for nidation. The 25 female rabbits were provided water and food ad libitum consisting of antibiotic-free granules. Animals were randomly assigned to four groups: 1) controls (saline serum inoculation); 2) C+ (*Escherichia coli* intrauterine inoculation); 3) R+ (saline serum inoculation + rolipram infusion); and 4) C+R+ (*Escherichia coli* inoculation + rolipram infusion). Pregnant rabbits in all groups (n = 25 for both groups) were treated with 200 mg/kg/day intravenous ceftriaxone (Rocephin; Roche, Meylan, France) via a catheter inserted into a marginal vein of the ear. Treatment began 6 h after intrauterine inoculation and lasted 4 days, until spontaneous delivery. Pregnant rabbits in all groups received dimethyl sulfoxide (DMSO), the rolipram diluent. DMSO was injected alone (0.02 mg + 20 ml of saline serum) or with rolipram (R6520; Sigma-Aldrich, Lyon, France), according to the experimental group. Posology of rolipram was 0.250 mg/kg in our study.
Evaluation of Cell Death

Cell death was assessed at day 0 using the DNA-specific dye Hoechst 33258 (Merck Biosciences, Fontenay-sous-Bois, France) at 1 mg/ml in Hanks’ balanced salt solution without phenol red on formalin-fixed paraffin sections. Sections were mounted with Prolong Antifade medium (Invitrogen, Carlsbad, CA). Fluorescence was observed with a fluorescence microscope (Axiocinst 200-M; Carl Zeiss, Göttingen, Germany) equipped with an ApoTome slider, which eliminates image blurring. Cells were visualized with a 63/1.4 X oil immersion lens. Image processing was performed using an AxioCam MR charge-coupled device camera and AxioVision 4.0 software (Carl Zeiss).

Evaluation of Lung Elastic Fiber Content

To evaluate lung parenchyma elastic fiber length content at day 5, lung sections were stained by the Weigert technique. The standard morphometric methods described above were then used (i.e., counting the number of points touching alveolar parenchyma (point counting) and the number of intersections between the glomerulate lines and the elastic fibers (mean linear intercept) (Weibel and Cruz-Orive, 1997).

Assessment of Vascularization

Vascularization was assessed at day 5 by immunohistochemistry using a monoclonal antibody against human CD31 (clone JC/70A; Dako North America, Inc.) as the primary antibody, diluted 1:50. The vascular development index was determined by counting the immunostained nuclei per alveoli in 25 fields of alveolar parenchyma per animal, using a Quantimet Q550 (Leica Microsystems).

Assessment of Total and Specific PDE Activity in Pup Lungs at Day 0

Whole lung tissues were homogenized in ice-cold hypotonic buffer (100 mM Tris-HCl, pH 7.4, 2 mM MgSO4, 2 mM EDTA, 50% glycerol, and 1 mM 2-β-mercaptoethanol supplemented with 2714 protease inhibitor cocktail; Sigma-Aldrich) using an all-glass homogenizer. Activities were measured with 1 mM McAMP as substrate (GE HealthCare, McGraw Park, IN).

Assessment of Total and Specific PDE Activity in Pup Lungs at Day 5

To evaluate lung elastic fiber length content at day 5, lung sections were stained by the Weigert technique. The standard morphometric methods described above were then used (i.e., counting the number of points touching alveolar parenchyma (point counting) and the number of intersections between the glomerulate lines and the elastic fibers (mean linear intercept) (Weibel and Cruz-Orive, 1997).

Assessment of Survival and Growth at Day 0 and Day 5

Survival. Five pregnant rabbits died between inoculation (day 28 of gestation) and delivery, three in group C + R + (days 29, 29, and 31) and two in group R + (both at day 31); however, mortality rates did not significantly differ between the four groups. No mother died after delivery. Among pups, intrauterine death was significantly increased in groups C + R + and R + compared with controls (p < 0.0001) and group C + (p < 0.03), and postnatal mortality was significantly increased in groups C + (p < 0.03) and C + R + (p < 0.04) relative to that in controls (Fig. 1). There were significant differences in survival of live-born pups at day 5 between controls (97 ± 2%) and groups C +, C + R +, and R + (31 ± 6, 22 ± 8, and 40 ± 5%, respectively, p < 0.0001, by log-rank test).

Body Weight Gain. (Table 1). Animals in the rolipram groups (R + and C + R +) and infection groups had significantly lower weight at birth. Five days after birth, infection and rolipram had no significant effect, but we observed a strong interaction between these two factors (F1,22 = 44.6, p = 0.000001). Weights were preserved between control animals and C + R + animals.

All maternal and fetal blood and tissue cultures remained sterile. We previously demonstrated in the same experimental model early fetal infection 8 h after bacterial challenge with 5 of 17 (29%) fetal blood positive cultures and 15 of 17 (88%) placental positive cultures with a mean quantitative value of 4.5 ± 1.4 log10 CFU · g tissue−1 (Gras-Le Guen et al., 2008).

Lung Morphometry at Day 0 and Day 5

Examination of postnatal lung sections under light microscopy at day 5 showed that infected rabbit lungs exhibited a diffuse and simplified lung structure with enlarged air spaces and fewer secondary septa, whereas infected rabbits treated antenatail with rolipram exhibited alveolar morphology similar to that of control animals. Quantification by morphometric analysis highlighted the following features (Tables 1 and 2).

Lung Volume. At day 0, no difference was found between the four groups for absolute lung volumes, but we noticed a significant effect of rolipram on specific lung volume (F1,22 = 7.1; p = 0.01). At day 5, absolute lung volume was significantly reduced in infection groups (F1,21 = 7.6; p = 0.01) with a strong interaction between infection and rolipram effects (F1,23 = 52.3; p = 0.000001), but we did not observe any effect of infection or rolipram on specific lung volume.

Alveolar Airspace. At day 0, we observed a significant effect of rolipram on all alveolar airspace parameters (volume...
density, absolute volume, and specific volume). Moreover, we noted a strong interaction effect on volume density ($F_{1,22} = 16.0; p = 0.0005$). At day 5, we reported some significant interaction effects on all alveolar airspace parameters. Rolipram seemed to have the same deleterious effect as infection on alveolar airspace, but preserved alveolar volume when it was used in infected animals.

**Alveolar Surface.** At day 0, we did not observe any significant effect of infection or rolipram on alveolar surface. At day 5, we reported a significant effect of rolipram on all alveolar surface parameters (area density, absolute area and specific area).

**Interstitium.** At day 0, specific volume was significantly increased in rolipram groups (C+R+ and R+) compared with that in both controls and group C+.

**Fig. 1.** Perinatal mortality from inoculation to birth of pups. Data are presented as relative value according to the time of death. Intrauterine death was significantly increased in groups C+R+ and R+ compared with that in both controls and group C+. 

**Table 1** Morphometric measurements at day 0

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>C+</th>
<th>C+R+</th>
<th>R+</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>56.68 ± 9.3</td>
<td>47.18 ± 4.5</td>
<td>32.18 ± 6.1</td>
<td>39.90 ± 2.7</td>
</tr>
<tr>
<td>Lung volume (cm³)</td>
<td>2.89 ± 0.19</td>
<td>2.61 ± 0.26</td>
<td>2.32 ± 0.68</td>
<td>2.36 ± 0.33</td>
</tr>
<tr>
<td>Specific lung volume (cm³/100 g)</td>
<td>5.23 ± 0.98</td>
<td>5.56 ± 0.62</td>
<td>7.09 ± 1.95</td>
<td>5.92 ± 0.73</td>
</tr>
<tr>
<td>Alveolar airspace</td>
<td></td>
<td></td>
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<tr>
<td>Volume density</td>
<td>0.66 ± 0.03</td>
<td>0.70 ± 0.02</td>
<td>0.64 ± 0.03</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>Absolute volume (cm³)</td>
<td>1.93 ± 0.11</td>
<td>1.66 ± 0.46</td>
<td>1.49 ± 0.46</td>
<td>1.59 ± 0.22</td>
</tr>
<tr>
<td>Specific volume (cm³/100 g)</td>
<td>3.50 ± 0.63</td>
<td>3.66 ± 0.78</td>
<td>4.54 ± 1.26</td>
<td>3.99 ± 0.47</td>
</tr>
<tr>
<td>Alveolar surface</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area density (cm²/cm³)</td>
<td>284 ± 34</td>
<td>225 ± 27</td>
<td>230 ± 84</td>
<td>223 ± 31</td>
</tr>
<tr>
<td>Absolute area(cm³)</td>
<td>822 ± 130</td>
<td>542 ± 193</td>
<td>520 ± 182</td>
<td>528 ± 108</td>
</tr>
<tr>
<td>Specific area (cm³/100 g)</td>
<td>1485 ± 337</td>
<td>1187 ± 336</td>
<td>1660 ± 838</td>
<td>1320 ± 233</td>
</tr>
<tr>
<td>Interstitium</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Volume density</td>
<td>0.28 ± 0.01</td>
<td>0.25 ± 0.04</td>
<td>0.28 ± 0.09</td>
<td>0.29 ± 0.07</td>
</tr>
<tr>
<td>Absolute volume (cm³)</td>
<td>0.82 ± 0.05</td>
<td>0.60 ± 0.19</td>
<td>0.64 ± 0.18</td>
<td>0.66 ± 0.11</td>
</tr>
<tr>
<td>Specific volume (cm³/100 g)</td>
<td>1.48 ± 0.26</td>
<td>1.33 ± 0.31</td>
<td>2.00 ± 0.60</td>
<td>1.68 ± 0.26</td>
</tr>
<tr>
<td>Airways</td>
<td></td>
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</tr>
<tr>
<td>Volume density</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Absolute volume (cm³)</td>
<td>0.06 ± 0.04</td>
<td>0.04 ± 0.03</td>
<td>0.05 ± 0.05</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Specific volume (cm³/100 g)</td>
<td>0.12 ± 0.09</td>
<td>0.10 ± 0.06</td>
<td>0.24 ± 0.15</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>Blood vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume density</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Absolute volume (cm³)</td>
<td>0.07 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.09 ± 0.06</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Specific volume (cm³/100 g)</td>
<td>0.14 ± 0.05</td>
<td>0.09 ± 0.01</td>
<td>0.29 ± 0.19</td>
<td>0.11 ± 0.06</td>
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</tbody>
</table>

Data are expressed as means ± S.D. Comparison results are expressed with the $F_{1,22}$ value accompanied by the corresponding $p$ value. Body weight: infection effect $F_{1,22} = 12.2, p = 0.0002$; rolipram effect $F_{1,22} = 27.7, p < 0.0001$; interaction $p > 0.05$. Alveolar airspace (volume density): rolipram effect $F_{1,22} = 11.7, p = 0.0002$; interaction $F_{1,22} = 16.0, p = 0.0005$. Alveolar airspace (specific volume): rolipram effect $F_{1,22} = 9.4, p = 0.008$. Blood vessels (volume density): interaction only $F_{1,22} = 6.8, p = 0.05$. Blood vessels (absolute volume): interaction only $F_{1,22} = 8.6, p = 0.007$. Blood vessels (specific volume): rolipram effect $F_{1,22} = 4.7, p = 0.04$; interaction $F_{1,22} = 7.8, p = 0.01$. All other comparisons were not statistically significant.
interaction effect on absolute volume \((F_{1,21} = 10.9; p = 0.003)\).

**Blood Vessels.** At birth, specific volume was significantly increased in rolipram groups, with an interaction effect on the three parameters. At day 5, we observed the same interaction effects on the blood vessels. Once again, morphometric parameters were decreased in group C+ but maintained in group C+R+ (Table 2). Moreover, microvasculation studied by immunohistochemical labeling was significantly altered in all groups compared with that in controls (Fig. 2A).

**Evaluation of Lung Elastic Fiber Content**

Morphological analysis of elastic fibers at day 5 showed a significant decrease in the number of alveolar fibers in the lungs of pups in groups C+, C+R+, and R+ versus that for controls (8 ± 4, 16 ± 5, and 4 ± 2 versus 40 ± 25, respectively; \(p < 0.05\)). The number of alveolar fibers tended to be less decreased in group C+R+ than in groups C+ and R+, but the difference was not significant (Fig. 2B).

**Assessment of Apoptosis and Cell Proliferation**

Apoptosis was present in the controls, and the group C+ parameters were not associated with an increase in cell apoptosis (Fig. 3A). A significant decrease was noticed in group R+ compared with that in both controls and C+R+ \((p < 0.001)\). For cell proliferation, an increase was noticed in the C+ group relative to controls \((p < 0.05)\) (Fig. 3B).

**Assessment of Inflammation in BAL Fluid at Day 0 and Day 5 and PDE4 Activity in Lung at Day 0**

**Mononuclear Cell Count in BAL Fluid.** No difference was found in the inflammatory cell count in BAL fluid, either at day 0 or at day 5. However, group C+R+ showed a trend, although not significant, toward an increase of mononuclear inflow (Table 3).

**PDE4 Activity in Lung.** Global and equivalent cAMP-phosphodiesterase activity was noted in all conditions at day 0. Mean PDE4 specific activity represented 48.7 ± 5.1% of the global cAMP-phosphodiesterase activity expressed in the pups’ lung for all conditions and did not change subsequent to the experimental condition (Fig. 4).

**Discussion**

In the present study, we report that inhibition of PDE4s by rolipram in rabbit pups exposed to chorioamnionitis preserved antenatal and postnatal alveolarization, without modifying the inflammatory response. However, we observed marked intrauterine growth retardation and a very high incidence of stillbirth in animals treated with rolipram, results not yet reported in this model. Rolipram is the prototype PDE4 selective inhibitor. PDEA4 enzyme is the main cAMP-metabolizing enzyme in immune and inflammatory cells, airway smooth muscle, and pulmonary nerves; its inhibition suppresses the recruitment and activation of several inflammatory cells (neutrophils, CD8 T cells, and macrophages) known to have a crucial role in the pathophysiological processes of bronchopulmonary dysplasia (Sanz et al., 2005; Hayes et al., 2010). In this context, we chose to test this new treatment in a previously described model of antenatal infection with subsequent impaired alveolarization in the rabbit (Gras-Le Guen et al., 2008). The detection of cAMP-phosphodiesterase activity in our study confirmed that PDE4s are expressed in pups’ lungs. The very short half-life of the molecule \((1.9 ± 0.6 \text{ h in the rabbit for a } 0.2 \text{ mg/kg dose})\) can probably explain the absence of significant changes in
the level of expression between the different groups, because the assays were possible only 6 to 12 h after birth, several hours after the last injection (Krause and Kühne, 1988). However, we can observe a tendency to a decreased expression in group C+R+ compared with that in group C+. The small posology that we used (0.25 mg/kg/day) is possibly responsible for this lack of difference but was imposed by the poor tolerance to higher doses observed in this model. However, these results confirm that PDE4 is a potential target in fetal and neonatal lungs.

Antenatal infusion of selective PDE4 inhibitors preserves alveologenesis in the context of chorioamnionitis. We observed conservation of specific interstitial, vascular, airway, and alveolar airspace volumes at day 0 and persistent effects at day 5 with some significant interaction effects on alveolar airspace, interstitium, airways, and vascular morphometric parameters. Rolipram effects seem clearly different in infected or in noninfected fetuses and newborns. These observations are consistent with the results of Woyda et al. (2009), using a different PDE4 inhibitor (cilomast) in a different experimental model of hyperoxia alveologenesis impairment in the mouse. However, the lack of a control group and the quality of morphometric determinations made the interpretation of the exact effects of PDE4 inhibition on disturbed alveolarization difficult (Méhats et al., 2009). Méhats et al. (2009), in the same model in rat, indicated that PDE4 inhibition with rolipram did not enhance alveolarization in rat pups exposed to hyperoxia and may directly affect alveolarization in rat pups exposed to room air. The authors suggested that this last point could be explained by the profound

Fig. 2. Microvascularization and elastoegenesis at day 5. A, immunohistochemical labeling for microvascularization. Monoclonal antibody against human CD31 was used. Data are percentage of labeled cells out of total parenchymal cells. Microvascularization was significantly decreased in groups C+, C+R+, and R+ compared with controls. Data are presented as means ± S.D. #, p < 0.01 compared with controls. B, specific elastic fiber length assessed by morphometry (centimeters per 100 g). Fiber length was significantly decreased in groups C+, C+R+, and R+ compared with that for controls. Data are presented as means ± S.D. #, p < 0.05 compared with controls.
effect of PDE4 inhibition on pups' weight gain that could interfere with normal alveolarization as much as antenatal infection interferes (Gras-Le Guen et al., 2008). In this infectious and inflammatory context, beneficial effects of PDE4 inhibition observed on alveologenesis in infected pups are possibly mediated by some specific anti-inflammatory mechanisms, whereas poor weight gain and poor alveolarization both could reflect poor animal nutrition.

Rolipram infusion was also concomitant with intrauterine growth restriction. In fact, this experimental model of antenatal infection has previously been associated with neonatal growth alteration, but only with a postnatal onset (Gras-Le Guen et al., 2008). In this present work, antenatal growth restriction is noted at birth in the two groups exposed to rolipram versus the infected group (C/H11001) and controls. However, postnatal growth restriction occurred as early as 5 days after birth in the group C+ but not in group C+R+ animals, in which rolipram seemed to have preserved postnatal growth restriction.

TABLE 3
Effects of antenatal infection, rolipram, or both on bronchoalveolar lavage cell count from day 0 to day 5
BAL cell count data are expressed as means ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 5</th>
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<tbody>
<tr>
<td></td>
<td>Controls C+ C+R+ R+</td>
<td>Controls C+ C+R+ R+</td>
</tr>
<tr>
<td>BAL cell count (×10⁴/ml)</td>
<td>1.3 ± 1.0 1.6 ± 1.7 2.4 ± 0.1 0.8 ± 0.2</td>
<td>0.9 ± 0.9 1.2 ± 1.1 5.1 ± 0.2 1.9 ± 1.2</td>
</tr>
<tr>
<td>PMNs (%)</td>
<td>0.51 ± 0.16 0.88 ± 0.45 0.91 ± 0.22 1.22 ± 0.67</td>
<td>2.07 ± 1.24 2.45 ± 1.56 3.66 ± 1.89 2.25 ± 1.12</td>
</tr>
</tbody>
</table>

PMN, polymorphonuclear leukocytes.
growth. However, de Visser et al. (2008) and Méhats et al. (2008) observed a weight loss in rat pups exposed to rolipram in an experimental model of hyperoxia-altered alveolarization. In humans, Fabbri et al. (2009) observed weight loss in the group of patients with chronic obstructive pulmonary diseases treated with roflumilast compared with a control group. To explain the weight loss, one hypothesis was that pups treated with rolipram were underfed because of side effects limiting the pregnant rabbits’ feeding. Rolipram is known to have adverse effects on the central nervous system that account for nausea, vomiting, and enhanced gastric acid secretions (Zeller et al., 1984; Barnette et al., 1995). However, mother rabbits did not lose weight during the delay from inoculation through delivery. Second, rolipram on its own increases lipolysis as described in an in vitro model of rat pups (Nakamura et al., 2004; Snyder et al., 2005). In addition, toxicological reports during preclinical studies demonstrated significant inflammation of the intestinal tract, suggesting pathological absorption of food (Larson et al., 1996; Dague et al., 2007). This mechanism would better explain the differences noted between group C and controls and group C+ versus groups C+R+ and R+ at birth, because animals treated with rolipram made up for the weight restriction as soon as rolipram infusion was stopped, and group C+R+ recovered normal weight at day 5 compared with controls.

Despite the fact that rolipram showed some protective effects on alveologenesis in the chorioamnionitis model, high rates of morbidity and mortality were noted in mothers and pups. Such a mortality rate had not been reported with pregnant rabbits in this experimental model (Guen et al., 2008). Hemodynamic effects on anesthetized and ventilated animals (decrease in pulmonary arterial pressure and systemic arterial pressure and increase in cardiac output) have been described, without the possibility of assessing morbo-mortality during these very short-term experiences (Schermuly et al., 1999). Cardiac toxicity has been described in rabbits by Shahid and Rodger (1989), who showed an in vitro increase in the intracellular concentration of cAMP that led to chronotropic effects on atrial muscle fibers. Nevertheless, this effect did not induce cardiac arrhythmias (Vaughan Williams, 1987). PDE4 inhibitors have been used in humans with a well described adverse event profile, and such severe or fatal effects have not been reported (Fabbri et al., 2009). The fatal adverse events in the rabbit are probably specific to this experimental model and constitute a limitation, as previously observed with corticotherapy (Pratt et al., 1999). In utero death of pups in groups C+R+ (52.1%) and R+ (46.8%) differed significantly from that for both controls (0.7%) and group C+ (18.9%), and the increase in mortality in group C+R+ compared with that in group C+ was probably related to the cumulative deleterious effects of chorioamnionitis and rolipram infusion. We hypothesized that the tocolytic effect of rolipram described by Schmitz et al. (2007) may have played a role in this high rate of in utero mortality; however, inoculation-delivery delay did not differ among the four groups. We also suggest that peripartum maternal behavior associated with maternal neglect could be implicated in the mortality of the pups after delivery as an indirect consequence of rolipram. Of interest, de Visser et al. (2008) and Méhats et al. (2008) observed a prolonged median survival in rat pups treated with rolipram only after birth. It seems clear that rolipram is implicated in the maternal and neonatal deaths in these studies, but further studies are needed to understand the mechanism involved in these severe adverse events.

PDE4 inhibitors appear to be a promising new class of anti-inflammatory drugs that have shown efficacy in the experimental model of chorioamnionitis-induced bronchopulmonary dysplasia described here. However, the conflicting results and side effects observed in different experimental models tempered the enthusiasm for this treatment. Further investigations are needed in several different models before conclusions can be made concerning the benefit/risk ratio of this treatment.

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Authorship Contributions
Participated in research design: Homer, Jarreau, Potel, and Gras-LeGuen.
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References


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