Improved Pulmonary Function by Acid Sphingomyelinase Inhibition in a Newborn Piglet Lavage Model

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Rationale: In acute inflammatory lung disease in newborn infants, exogenous surfactant only transiently improves lung function. We hypothesized that the transient nature of this protection is in part explained by elevated acid sphingomyelinase (a-SMase) activity that may inactivate surfactant and promote proinflammatory responses. Objectives: We investigated the intermediate-term effects (12 h) of a-SMase inhibition in a neonatal piglet model of repeated airway lavage by intratracheal use of the a-SMase inhibitor imipramine, together with exogenous surfactant as a carrier substance. Methods: After surfactant washout and induction of pulmonary inflammation, lung function was monitored over 24 hours of mechanical ventilation and followed by ex vivo analyses. In addition, we studied the effect of lipopolysaccharide inhalation in a-SMase-deficient mice at 48 hours. Measurements and Main Results: Surfactant washout increased both pulmonary a-SMase activity and ceramide content; this was attenuated by surfactant and prevented in the surfactant plus imipramine group. Compared with surfactant alone, PaO2 dynamic compliance, and extravascular lung water were improved in the final 12 hours in the surfactant plus imipramine group. At 24 hours, lavage fluid leukocyte counts and IL-8 concentrations decreased, and physical surfactant film properties improved. In the mouse model at 48 hours, a-SMase-deficient mice showed reduced pulmonary ceramide levels and attenuated leukocyte influx into the alveolar space. Conclusions: We conclude that stabilization of exogenous surfactant by adding imipramine to create a "fortified surfactant preparation" improves lung function in a clinically relevant piglet model, and that this effect can be attributed to the inhibition of a-SMase as evidenced in the mouse model.

Keywords: acute neonatal inflammatory lung injury; ceramide; imipramine; surfactant; acid sphingomyelinase–deficient mice

In acute inflammatory lung disease in newborn infants, gas exchange and lung function are impaired as a result of alveolar atelectasis, cell infiltration, and the accumulation of proteinaceous extra- and intraalveolar fluid in lung tissue (1). Prevention of alveolar flooding depends on intact endothelial and epithelial barrier function, and on the maintenance of an intact surfactant film. Rauvala and Hallman (2) first described increased concentrations of glycosylated ceramide in the bronchoalveolar lavage fluid (BALF) of adult patients with acute lung injury and the negative impact of such glycolipids on the physical properties of surfactant films.

Ceramide is generated from sphingomyelin by sphingomyelinase activity, or by de novo synthesis by the enzyme ceramide synthase (3). Ceramide (generated by a tumor necrosis factor–α–inducible secretory acid sphingomyelinase [a-SMase]) inhibits the synthesis of disaturated phosphatidylcholine, the major phospholipid component of surfactant (4), by inhibiting cholinephosphate cytidylyltransferase (5). We proposed an alternative role of a-SMase and ceramide synthesis in the development of acute lung injury and demonstrated that this pathway increases endothelial vascular permeability (6, 7). Thus, ceramide appears to promote edema formation by at least two mechanisms, that is, by altering vascular permeability and by impairing surfactant function. In addition, ceramide generated by a-SMase is involved in signaling pathways leading to apoptosis in response to tumor necrosis factor (TNF) (8), CD95 (9), TNF-related apoptosis-inducing ligand (10), LPS (11), and γ irradiation (12). The a-SMase pathway was discussed by Mercier and Dinh-Xuan (13) as a possible therapeutic target to improve lung function in acute inflammatory lung disease in newborns.

The antidepressant imipramine is known to suppress a-SMase activity in various cell types within 0.5–2 hours (14). This effect lasts for 2–3 weeks (15). Imipramine is not a specific a-SMase inhibitor but interferes with the binding of a-SMase to the lipid bilayers, and thereby displaces the enzyme from its membrane-bound substrate (16).

The aim of the present study was to investigate whether inhibition of a-SMase by a single dose of intratracheal imipramine together with exogenous surfactant as a carrier results in intermediate-term (>12 h) improvement in gas exchange, lung...
function, and pulmonary edema in a neonatal piglet model of repeated airway lavage, or whether any beneficial effect is short-lived, as seen in most models of acute lung injury covering 12–24 hours of observation. Our clinical outcome parameters were gas exchange, compliance of the respiratory system (Crs), and extravascular lung water (EVLW). After 24 hours, we determined pulmonary a-SMase activity, ceramide concentration, alveolar cell counts, chemokine concentrations, translocation of nuclear factor (NF)-κB to the nucleus of pulmonary cells, and surface tensions in surfactant films. In addition, to confirm the contribution of a-SMase to acute inflammatory lung injury, we studied inflammatory responses in a-SMase–deficient and wild-type mice 48 hours after inhalation of *Pseudomonas aeruginosa* LPS.

**METHODS**

**Animal Preparation and Clinical Care**

The experimental protocol was approved by the local review board for the care of animal subjects. Twenty-four male piglets were studied between Days 2 and 5 of life. See the online supplement for additional details on animal preparation and clinical care.

**Mechanical Ventilation and Airway Lavage**

The piglets were ventilated with a fraction of inspired oxygen (FIO2) of 0.5, and a positive end-expiratory pressure (PEEP) of 6 mbar. Peak inspiratory pressure (PIP) was adjusted to keep tidal volume (VT) at 7 ml/kg, until PaO2/FIO2 decreased to no more than 92 mm Hg, and a PIP of at least 19 mbar was needed to maintain VT at 7 ml/kg (for more detail, see the online supplement).

**Experimental Protocol**

After airway lavage at baseline the piglets were randomized to one of the following three groups:

- A control group receiving an air bolus
- Two intervention groups (Surf and Surf + Imi) receiving porcine surfactant (100 mg/kg) without or with 5 mg of imipramine admixed to surfactant

The surfactant preparation used was a freeze-dried surfactant preparation isolated from minced porcine lungs.

**Measurements of Gas Exchange, Hemodynamic Parameters, and Lung Function**

To assess gas exchange we measured Pao2 and Paco2 every 1–2 hours for calculation of a ventilation efficiency index [VEI = 3,800/(PIP – PEEP) × f × Pao2Cv], where f is respiratory frequency. Cardiac output monitoring with EVLW determination was performed before and after acute lung injury, and 1, 3, 6, 12, 18, and 24 hours after intervention. To calculate FRC, the alveolar portion of the tidal volume (VA), VT, and dynamic Crs, we used the nitrogen washout method for lung volume measurements, and the single-breath least-squares method for lung mechanics as previously described (17) (for more detail, see the online supplement).

**Cytokine Assays**

IL-8 and leukotriene B4 (LTB4) concentrations from BALF were determined with porcine Quantikine ELISA kits (R&D Systems, Wiesbaden, Germany). LTB4 concentrations were measured by immunoassay (R&D Systems).

**Ex Vivo Lung Processing and NF-κB Measurements**

Small tissue specimens were taken to determine dry:wet ratios. Additional specimens were frozen at –80°C for NF-κB electrophoretic mobility shift assay.

**Surface Tension Measurements**

Surface tension measurements were performed as previously described (18).

**Acid Sphingomyelinase and Ceramide Measurements**

a-SMase activity was determined by a modified micellar *in vitro* assay. Ceramide levels were determined by two-dimensional charring densitometry (for more detail, see the online supplement).

**a-SMase Knockout Mouse Model of LPS Inhalation**

The genetic background of the mice was C57BL/6 × 129/SVEV, with the a-SMase knockout (KO) genotype inherited as an autosomal recessive trait (19). All a-SMase–deficient (KO/KO) or competent (wild-type/wild-type) mice were subjected to nasal inhalation of 10 μg of *Pseudomonas aeruginosa* serotype 10 LPS (Sigma-Aldrich, St. Louis, MO). In the intervention groups, 5 or 50 μg of imipramine, respectively, was given intraperitoneally. Mice were monitored for 48 hours and killed by sevoflurane narcosis. The lungs were lavaged and dissected from the thorax for determination of a-SMase activity and ceramide content, using the methods described for the piglet model.

**Statistical Methods**

To correct for known deviations from normality, before analysis all cell count and cell percentage data were subjected to square root and arcsine transformations, respectively. Univariate data were checked for heteroscedasticity and, when necessary, transformed by the Box-Cox transformation, before they were analyzed by univariate analysis of variance. Repeated measurement data were analyzed by mixed model analysis. Both the analysis of variance and the mixed model analysis were followed by individual t tests only when the omnibus tests showed a significant treatment effect. P < 0.05 was considered to be significant. The transformations were used for the statistics, but are not shown in the figures. All data represent means ± SEM. The analyses were performed with JMP 6.0 and SAS 9.1 software (SAS Institute, Cary, NC).

**RESULTS**

**Comparability of Study Groups**

Twenty-four piglets weighing 2.3 ± 0.1 kg were studied in pairs of 2, according to the randomization protocol after airway lavage. One piglet in the Surf + Imi group was excluded because of prevalent airway infection, and one control group piglet was excluded because of insufficient surfactant removal from the airways due to a left bronchial stenosis. There were no significant differences in body weight, the number of lavages performed, loss of lavage fluid in the airways, final PaO2/FIO2, and PIP between the three groups at baseline, the latter two parameters being used as the endpoints for the lavage procedure (Table 1). At baseline (completion of airway lavage) there were no significant differences between the three groups for PaO2/FIO2, VEI, Crs, FRC, and EVLW (Table 1).

**a-SMase and Ceramide**

Twenty-four hours after lavage, both a-SMase activity and ceramide levels were about doubled in the lung tissues (Figure 1). This increase was attenuated by surfactant treatment (Surf) and completely abolished in those animals that received both surfactant and imipramine (Surf + Imi). Serum concentrations of a-SMase and ceramide showed similar distributions as measured in pulmonary tissue.

**Clinical Parameters**

PaO2/FIO2 and VEI differed between the three groups throughout the entire study period (Figure 2). In the second half of the experiment, oxygenation and ventilation worsened in the
surfactant-treated animals (Surf), but remained stable in the Surf + Imi group.

Crs, FRC, and EVLW showed significant differences between 12 and 24 hours only (Figure 3). Whereas surfactant alone improved none of these parameters, additional administration of imipramine significantly improved Crs and EVLW compared with both the control and Surf groups. Compared with the control group, the FRC was improved by Surf + Imi, but not by surfactant alone. As stipulated in the study design, no differences were observed in VT by hourly ventilator adjustments to approximate 7 ml/kg.

### Leukocytes in Blood and BALF

Similar results for complete cell count and the percentage of polymorphonuclear (PMN) leukocytes were found in the blood of all three groups. In contrast, bands at 24 hours differed significantly (control: 11.7 ± 2.9%; Surf: 8.7 ± 2.0%; Surf + Imi: 4.2 ± 1.0% [P < 0.05]). Repeated airway lavage resulted in a substantial increase in blood leukocyte counts compared with prelavage values (average PMN leukocyte count for all piglets before lavage: 10.465 ± 753 cells/μl; at baseline: 17.062 ± 2.001 cells/μl). Imipramine administration tended to increase this effect at 3 hours (control: 14.535 ± 3504 cells/μl; Surf: 13.357 ± 2.663 cells/μl; Surf + Imi: 23.971 ± 3.699 cells/μl). At 24 hours PMN counts were as follows: control: 11.207 ± 1.655 cells/μl; Surf: 8.443 ± 1.769 cells/μl; Surf + Imi: 8.371 ± 1.032 cells/μl (P = NS). Percentage of PMN leukocytes did not differ between the groups.

In all groups BALF cell counts were similar before acute lung injury caused by repeated airway lavage, and were increased at the end of the experiment (Figure 4). The total cell count was reduced in the Surf + Imi group only (Figure 4A). Both surfactant and imipramine slightly, but significantly, attenuated PMN and mononuclear cell counts in the BALF; the effect in the Surf + Imi group tended to be more pronounced (Figures 4B and 4C).

### Cytokine Assays

The lavage fluid concentrations of IL-8 (Figure 5A) and LTB₄ (Figure 5B) were increased after 24 hours of mechanical ventilation in the injury control group. These mediator levels were decreased in both the Surf group and the Surf + Imi group, but this was significant for IL-8 only.

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**TABLE 1. COMPARABILITY OF STUDY GROUPS AT BASELINE**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>Surf (n = 8)</th>
<th>Surf + Imi (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>2.2 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Lavages, n</td>
<td>22 ± 2</td>
<td>20 ± 2</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>Loss of lavage fluid, %</td>
<td>9 ± 1</td>
<td>10 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Loss of lavage fluid, ml/kg</td>
<td>62 ± 6</td>
<td>59 ± 7</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>Pao₂/Fio₂, mm Hg</td>
<td>88 ± 2</td>
<td>79 ± 3</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>PIP, mbar</td>
<td>25.5 ± 1.1</td>
<td>24.7 ± 1.3</td>
<td>24.8 ± 1.2</td>
</tr>
<tr>
<td>VEI, 3,800/(PIP – PEEP)</td>
<td>0.09 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

*Definition of abbreviations: EVLW = extravascular lung water; f = respiratory frequency; Fio₂ = fraction of inspired oxygen; PEEP = positive end-expiratory pressure; PIP = peak inspiratory pressure; VEI = ventilation efficiency index.

There were no significant differences between the three groups at baseline. PIP (>19 mbar) and PaO₂/FIO₂ (>85 mm Hg) were also used as endpoints of lavage. Data represent means ± SEM.
Wet Lung Weight: Dry Lung Weight Ratio

Wet lung weight: dry lung weight ratios from small lung specimens were as follows: control, 9.2 ± 0.2; Surf, 8.7 ± 0.3; Surf + Imi, 8.0 ± 0.1 (P = 0.08).

NF-κB Electrophoretic Mobility Shift Assay

Translocation of NF-κB to the nucleus of pulmonary cells differed between the three groups (Figure 6); both surfactant-treated piglets and piglets treated with surfactant plus imipramine showed reduced NF-κB levels compared with control group piglets.

Surface Tension

Surface tensions from surfactant films differed between the three groups after 24 hours of mechanical ventilation (Figure 7). The combined surfactant and imipramine treatment (Surf + Imi) reduced both minimal and maximal surface area tensions, whereas at minimal surface area surfactant treatment alone (Surf) did not improve control results.
Assessment of Toxic Side Effects in Imipramine-treated Piglets

No episode of arrhythmia or severe arterial hypotension was observed in imipramine-treated piglets. When comparing heart rate and blood pressure 18 hours after intervention (18 h is the approximate half-life of imipramine in neonates), we monitored the following parameters: (1) heart rate: control, 205 ± 10 beats/minute; Surf, 206 ± 4 beats/minute; Surf+Imi, 204 ± 7 beats/minute ($P = 0.98$); (2) systolic blood pressure: control, 80 ± 2 mm Hg; Surf, 82 ± 2 mm Hg; Surf+Imi, 97 ± 7 mm Hg ($P = 0.03$); (3) diastolic blood pressure: control, 44 ± 3 mm Hg; Surf, 45 ± 2 mm Hg; Surf+Imi, 52 ± 4 mm Hg ($P = 0.21$).

Imipramine and desipramine (important active metabolite) blood concentrations at 18 hours were as follows: imipramine, 82 ± 13 mg/L (range, 32–146 mg/L); desipramine, 22 ± 3 µg/L (range, 11–34 µg/L).

**Figure 4.** Cell concentrations and percentages in bronchoalveolar lavage fluid (BALF) of the lung before lavage and after 24 hours of mechanical ventilation. (A) Total cell count; significant difference in Surf+Imi group when compared with the control group ($*P < 0.05$) and the Surf group ($*P < 0.05$); (B) polymorphonuclear (PMN) leukocytes: $***P < 0.001$ versus control; (C) Mononuclear cells. Data represent means ± SEM.

**Figure 5.** (A) IL-8 and (B) leukotriene B₄ (LTB₄) concentrations in BALF after 24 hours of mechanical ventilation. Data represent means ± SEM. $***P < 0.001$ versus control.

**Figure 6.** Nuclear factor (NF)-κB electrophoretic mobility shift assay of pulverized lung tissue after 24 hours of mechanical ventilation. Shown are arbitrary units (control mean, 1.0) for NF-κB activity comparisons between groups. Data represent means ± SEM. $*P < 0.05$ versus control; $***P < 0.001$ versus control.

**Figure 7.** Surfactant surface tension from BALF after 24 hours of mechanical ventilation at minimal (12.8 cm²) and maximal (64 cm²) surface area of a modified Wilhelmy balance. Data represent means ± SEM. $*P < 0.05$ versus control; $**P < 0.01$ versus control.
a-SMase KO Mouse Model of LPS Inhalation

To confirm the role of a-SMase for the development of acute inflammatory lung injury at later time points, we studied wild-type and a-SMase–deficient mice 48 hours after inhalation of *Pseudomonas aeruginosa* LPS and treatment with two different imipramine doses in the intervention groups. Table 2 summarizes all data from the mouse experiment with age, weight difference at 48 hours after intervention, cell count in BALF, percentage of PMN leukocytes in BALF, a-SMase activity, and ceramide content in lung tissue. Weight loss decreased in KO mice, when compared with the wild-type control group (P < 0.05). a-SMase KO mice showed significantly lower cell counts 48 hours after LPS inhalation compared with wild-type control mice (P < 0.01). Complete cell counts were dependent on imipramine dose, but stayed unchanged in KO mice. Relative PMN leukocyte counts were slightly, but significantly, reduced. As expected, a-SMase activity in KO mice was virtually absent, and pulmonary ceramide levels were lower than in wild-type mice. There was a dose-dependent reduction in pulmonary ceramide by imipramine treatment in wild-type mice.

### DISCUSSION

#### Summary

Repeated airway lavage is a widely used model of acute lung injury in neonatal animals, representing both surfactant deficiency and pulmonary inflammation. Although surfactant replacement therapy is effective in the early phase of the lung injury, its beneficial effects wear off later than 12 hours after intervention, as also became obvious in the present study. One explanation for this finding is that the lavage process not only disrupts the alveolar surfactant homeostasis, but also incites repetitive lavage increased pulmonary a-SMase activity and proinflammatory mechanism (21). In line with this, we observed that repetitive lavage increased pulmonary a-SMase activity and ceramide production. Remarkably, therapeutic intervention with the a-SMase inhibitor imipramine admixed to a porcine surfactant preparation significantly improved P_{AOU}, Crs, and EVLW during the second half of the experiments (from 12 to 24 h) compared with surfactant administration alone. Surfactant plus imipramine treatment lowered leukocyte influx into the alveolar space and tended to be more effective than surfactant alone. Further—albeit nonsignificant—improvements by imipramine treatment were seen in VEL, FRC, IL-8, LTB₄, NF-κB translocation, and surfactant surface tension. These findings extend the critical role of the a-SMase pathway during pulmonary inflammation to the lavage model of acute neonatal lung injury. More importantly, our findings show that surfactant fortification with imipramine is beneficial for at least 24 hours (intermediate-term effect). The drug-related effect of imipramine as a potent inhibitor of a-SMase activity in another type of acute lung injury was corroborated in an a-SMase–deficient murine KO model, demonstrating a dose-dependent reduction in its activity in wild-type mice, while a-SMase activity stayed at a virtually absent level in a-SMase KO mice regardless of imipramine administration at two different doses.

#### Role of Imipramine as Nonspecific a-SMase Inhibitor in Lung Metabolism

The reduction in a-SMase activity and ceramide levels in lung tissue of piglets in the Surf+Imi group at 24 hours indicates that (1) imipramine is not irreversibly bound (22) to surfactant components (as is also suggested by the lack of change in surface tension measurements when adding imipramine to surfactant *in vitro*; data not shown); (2) imipramine is well distributed in the lung, using surfactant as a carrier; (3) the cell permeability of imipramine is sufficiently high to enter cells *in vivo*; (4) imipramine indeed abolishes a-SMase activity, as claimed in the hypothesis; and (5) suppression of a-SMase activity in lung tissue lasts for at least 24 hours (15) and preserves surfactant from secondary functional loss through increasing ceramide concentrations (2).

Imipramine interacts with dipalmitylophosphatidylcholine via two binding sites with differing affinities. Compared with other amphiphilic drugs, such as amiodarone or chlorpromazine, its binding potency to dipalmitylophosphatidylcholine is classified as mild (23). In 1983, Grabner and Meerbach observed that imipramine increases phospholipid levels in alveolar macrophages in rats to an extent that they could explain only by an accumulation of phospholipids through impaired clearance (24). They later also described accelerated maturation of type II pneumocytes in fetal rat lungs after three doses of imipramine and cesarean section on Days 20, 21, and 22 of gestation (25); in lung specimens, they observed substantially better aeration compared with nontreated controls. In an isolated perfused rabbit lung, Eling and coworkers (26) demonstrated a nonfluxable pool consisting of approximately 30% infused imipra-

### TABLE 2. ACID SPHINGOMYELINASE KNOCKOUT MOUSE MODEL OF LIPOPOLYSACCHARIDE INHALATION

<table>
<thead>
<tr>
<th></th>
<th>WT-Control (n = 7)</th>
<th>WT-5 µg Imi (n = 7)</th>
<th>WT-50 µg Imi (n = 8)</th>
<th>KO-Control (n = 8)</th>
<th>KO-5 µg Imi (n = 7)</th>
<th>KO-50 µg Imi (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at intervention, d</strong></td>
<td>93 ± 8</td>
<td>97 ± 13</td>
<td>87 ± 11</td>
<td>78 ± 9</td>
<td>83 ± 0.2</td>
<td>74 ± 8</td>
</tr>
<tr>
<td><strong>Weight difference at 48 h, g</strong></td>
<td>-3.9 ± 0.3</td>
<td>-3.9 ± 0.4</td>
<td>-3.4 ± 0.3</td>
<td>-3.4 ± 0.3</td>
<td>-3.2 ± 0.1</td>
<td>-3.1 ± 0.2</td>
</tr>
<tr>
<td><strong>Cells in BALF, cells/µl</strong></td>
<td>201 ± 43</td>
<td>166 ± 29</td>
<td>84 ± 19</td>
<td>128 ± 32</td>
<td>110 ± 25</td>
<td>121 ± 21</td>
</tr>
<tr>
<td><strong>PMN leukocytes in BALF, %</strong></td>
<td>88 ± 1</td>
<td>89 ± 1</td>
<td>89 ± 2</td>
<td>80 ± 4</td>
<td>78 ± 7</td>
<td>78 ± 6</td>
</tr>
<tr>
<td><strong>a-SMase, nmol/mg/h</strong></td>
<td>20.0 ± 1.1</td>
<td>16.3 ± 0.9</td>
<td>13.8 ± 0.2</td>
<td>0.31 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td><strong>Ceramide, pmol C₁₈/mg lung tissue</strong></td>
<td>1,207 ± 50</td>
<td>842 ± 68</td>
<td>706 ± 67</td>
<td>755 ± 50</td>
<td>762 ± 41</td>
<td>819 ± 36</td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** a-SMase = acid sphingomyelase; BALF = bronchoalveolar lavage fluid; Imi = imipramine; KO = knockout; PMN = polymorphonuclear; WT = wild type.

* Data represent means ± SEM.
* Differences not significant.
* Differences between wild-type mice not significant; difference between wild-type control and the three KO groups significant: P < 0.05.
* Differences between wild-type mice significant (WT-control vs. WT-50 µg Imi, P < 0.05); difference between wild-type control and all three KO groups significant (P < 0.01).
* Differences between all wild-type groups versus all three KO groups significant (P < 0.01).
* Differences between wild-type mice significant (WT-control vs. WT-5 µg Imi, P < 0.05; WT-control vs. WT-50 µg Imi, P < 0.001).
* Differences between wild-type mice significant (WT-control vs. WT-5 µg Imi, P < 0.01; WT-control vs. WT-50 µg Imi, P < 0.001).
mine after an 8-minute infusion; they suggested compartmentalization of an imipramine–surfactant complex within lamellar bodies resulting from the interaction of phospholipids and imipramine. This latter finding was confirmed by Joshi and coworkers in lung lamellar bodies from Sprague-Dawley rats (27). Others (22) claimed a preferential accumulation of imipramine in lysosomes of many organs with the following order of deposition: lung, fat, heart, kidney, brain, gut, muscle, and bone. Plasma ceramide levels are increased in adult septic patients and correlate with mortality (28).

Effects of Imipramine on Pulmonary Inflammation

The positive effects of imipramine on lung function in this study may be explained by its beneficial effects on surfactant function aided by the salutary attenuation of pulmonary inflammation. Imipramine attenuated pulmonary edema (as indicated by a decrease in EVLW in the second half of the experiment). The fact that the wet:dry ratio was not significantly improved may suggest that the EVLW is a more sensitive measurement, or that the wet:dry ratio is affected by other factors such as infiltrating cells or clearance by the lymphatics. We also observed a tendency toward decreased pulmonary leukocyte influx and IL-8 levels. As these differences were not significant, inhibition of a-SMase does not seem to play an exclusive role in the development of pulmonary inflammation. Nonetheless, similar observations were made in the LPS mouse model, in which we observed less inflammatory cell influx into the alveolar space. These findings are in line with the intermediate-term effect that a-SMase has on pulmonary inflammation in this piglet model of acute inflammatory lung injury. In addition, the similarity of the findings in the piglet lavage model and in the mouse LPS inhalation model further supports the contention that the beneficial effect of imipramine is related to its effect on a-SMase activity. Unfortunately, the repeated lavage model is not feasible in mice, so that as an alternative we had to resort to the LPS model. Our findings corroborate observations in other models in which inhibition of a-SMase attenuated edema formation in response to platelet-activating factor, LPS, and acid instillation (6) and prevented LPS-induced expression of adhesion molecules in the pulmonary vasculature and leukocyte infiltration (11, 29). Ceramide-dependent IL-8 production was observed in various human cell lines such as Helicobacter pylori–exposed gastric cancer cells via activation of NF-κB (30), and in endometrial stromal cells after treatment with C2– and C3-ceramide by the intermediate action of IL-1α (31).

The molecular mechanisms behind these antiinflammatory effects remain poorly defined and seem to be generated by multiple signal transduction pathways, as suggested above. Because the antiedematogenic effects of imipramine also occurred in blood-free perfused lungs challenged with platelet-activating factor (6), this effect of imipramine should be independent of its effect on PMN leukocyte activation. Besides potential roles of ceramide as a secondary messenger—a consideration to some extent supported by equivalent distribution of ceramide content in pulmonary tissue and serum between the control, Surf, and Surf+Imi groups—a more general explanation might be that ceramide becomes enriched in lipid rafts, which may play a crucial role in inflammatory signaling (32).

Effect of Surfactant on Ceramide Generation and Inflammation

Another important finding of this study is the effect of lavage and of exogenous surfactant on a-SMase activity and ceramide generation in lung tissue that, to the best of our knowledge, has not been shown before in a translational model. The fact that this effect was already attenuated by surfactant alone (without additional imipramine) suggests that pulmonary stress–induced ceramide generation may also be self-limited by endogenous surfactant production. Indeed, many forms of stress such as γ irradiation (12), ultraviolet light (33), or infection with pathogens are known to increase ceramide production. How this stress is transduced in the present model remains speculative.

Ceramide and NF-κB Interdependence

The results of our study suggest that ceramide production and the increase in NF-κB translocation to the nucleus of pulmonary cells are unrelated. Imipramine appears to exert a promoting effect on NF-κB translocation when compared with the effect of surfactant only (Figure 6). Using a translational model, our results do not reveal a causative relationship or a possible chain of events being responsible for these unrelated occurrences. Schütze and coworkers (34) first suggested a rapid induction of NF-κB activity by a-SMase–induced ceramide generation in U937 cells, a concept later backed up by the finding of reduced levels of phosphorylated IκBα (inhibitor of NF-κB, isoform α) and degradation of both IκBα and IκBβ in the transformed T-cell line TpM(803) (35). On the other hand, there is evidence that ceramide generated in response to TNF-α or Fas activation is not involved in NF-κB activation in Jurkat (acute lymphocytic T-cell leukemia) cells (36), and that neither a-SMase nor ceramide induce NF-κB DNA binding, loss of IκBα, or NF-κB–dependent transcription in pulmonary A549 cells (37). It should be noted that in this study NF-κB activation was measured at 24 hours, and thus measurements may not correlate with the maximum activation in the very early stress response.

Reduced Surfactant Inactivation by Fortification

Significantly lower surfactant surface tensions at minimal and maximal surface, when compared with the control group, were achieved only by imipramine treatment (Surf+Imi group), which reveals an important physiological effect of this treatment modality. Our data also show that endogenous surfactant was produced by control group piglets within 24 hours of mechanical ventilation, and that imipramine is able, to some extent, to prevent the mixture of endogenous and exogenous surfactant from being degraded.

Our findings are in line with the results of Rauvala and Hallman, who reported increased minimal surface tensions of surfactant films by adding various types of ceramide to natural human surfactant (2). Fortified surfactant preparations have been a subject of interest for many years because a variety of severe lung diseases with inflammation (such as acute respiratory distress syndrome) tend to destroy exogenous surfactant once it is given into the airways (38). One relevant mechanism is capillary leakage and transmission into lung tissue of large-molecule peptides, which are known to cause surfactant degradation or inhibition (39). The approach of making exogenous surfactant less vulnerable to inflammatory attack within the airways was successful to some degree in experiments with an admixture of ionic and nonionic polymers such as hyaluronan (40), dextran (41), and polyethylene glycol (42), all of which share the chemical property of binding water.

Limitations of This Study

Dosage. The imipramine dose of 5 mg dissolved in surfactant for an average piglet weighing 2.3 kg (i.e., 2.2 mg/kg) was derived from clinical experience with pediatric doses of less than 5 mg/kg per os (43) to avoid toxic side effects such as cardiovascular compromise. We monitored higher systemic blood pressures in imipramine-treated piglets, a (nontoxic) side effect attributable...
to inhibition of serotonin and noradrenaline uptake (44), which may also be responsible for the increase in blood leukocyte counts in imipramine-treated piglets because of the sympathetic stimulus leading to leukocyte separation from blood vessel walls. Lower doses may be equally effective in lowering a-SMase activity, but the high volume of distribution of imipramine (23 ± 8 L/kg in adult individuals) prompted us to use this high dose.

Drug interaction. Intermittent or permanent binding of imipramine with surfactant components cannot be excluded.

Specificity. The tricyclic antidepressant imipramine is not a specific a-SMase inhibitor, and at present it is not intended to be used in any medical condition in neonates. Other substances such as D609 (6) are available or are being developed and may be worth investigation for this specific indication.

Animal model. The airway lavage model in the newborn piglet may not exactly represent the physiological and histologic situation of a newborn infant with acute inflammatory lung disease. Airway lavage causes widespread atelectasis alternating with areas of well-expanded or even hyperexpanded alveoli, necrosis and desquamation of airway epithelium, accumulation of edema fluid, hyaline membranes, nonspecific pneumonia, interstitial and intraalveolar accumulation of macrophages, PMN leukocytes, bone marrow–derived precursor cells, and areas of interstitial and intraalveolar hemorrhage (17, 45). Cell infiltration, regional differences in lung expansion, and pulmonary edema more closely mimic acute lung injury after an infectious process, whereas atelectasis and proteinaceous alveolar edema without cell infiltration are more comparable to the respiratory distress syndrome of the premature infant as shown by Jackson and coworkers in a premature monkey model (46). Recruitment of atelectatic areas in an adult porcine model of acute lung injury, as an example, is more successful in the airway lavage model than in the oleic acid injection model despite comparable limitations in gas exchange (47), a finding that underlines the impact of even subtle differences in lung injury on success in a variety of therapeutic interventions.

Minor differences in lung injury. Minor differences in lung injury by repeated airway lavage occurred despite the use of two clinical parameters (PaO2/FIO2 ≤ 92 mm Hg, PIP ≤ 19 mbar) as endpoints of the lavage procedure. These differences were to some extent augmented, but not significantly, in FRC levels.

Conclusions

Acute lung injury induced by repeated airway lavage was associated with activation of a-SMase and increased pulmonary ceramide levels. Inhibition of this process by admixture of imipramine to surfactant improved gas exchange, lung function, and extravascular lung water. Imipramine and surfactant synergistically suppressed a-SMase activity in lung tissue and reduced ceramide generation almost to a level seen in control piglets that were neither lavaged nor ventilated (Figure 1A). Surfactant fortification by means of early suppression of ceramide generation resulted in intermediate-term improvement in lung function over 24 hours of mechanical ventilation, which exceeds the beneficial effects of most other interventions shown in comparable newborn animal models of acute inflammatory lung injury. The specificity of imipramine as a potent inhibitor of a-SMase activity was corroborated by the fact that we obtained identical data in a murine a-SMase KO model. We speculate that fortification of exogenous surfactant by specific a-SMase inhibitors, as suggested by Mercier and Dinh-Xuan (13), might be a valuable therapeutic concept for the treatment of severe inflammatory lung disease of newborns and young infants.

Conflict of Interest Statement: P.v.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.-F.G.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.W.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.F.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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