Pulmonary surfactant synthesis after unilateral lung injury in mice

Giulia Lamonica,1 Maria Amigoni,2 Luca Vedovelli,3 Vanessa Zambelli,4 Margherita Scanziani,2 Giacomo Bellani,2,4 Alice Grassi,4 Manuela Simonato,5 Virgilio P. Carielli,6 and Paola E. Cogo7

1Department of Woman and Child Health, University of Padova, Padova, Italy; 2Department of Emergency, S. Gerardo Hospital, Monza, Italy; 3Department of Pharmacology and Anesthesia, University of Padova, Padova, Italy; 4Department of Health Science, University of Milano-Bicocca, Monza, Italy; 5Department of Medicine, Anesthesia and Critical Care of Padova, Padova, Italy; 6Department of Clinical Sciences, Polytechnic University of Marche and Ospedali Riuniti, Ancona, Italy; and 7Pediatric Cardiac Anesthesia/Intensive Pediatric Cardiology and Cardiac Surgery, Bambino Gesù Children’s Hospital, Rome, Italy

Submitted 21 December 2012; accepted in final form 4 December 2013

Abstract

Aspiration pneumonitis (AP) is due to an inhalation of the stomach acid content into the lungs, and it is a leading cause of acute respiratory failure during major surgery (2). More widely, AP occurs during a depressed level of consciousness (sedation, trauma, alcohol intoxication, seizures, drug abuse) or during an impaired gag reflex (i.e., during nasogastric or endotracheal intubation). As a consequence of acid aspiration, acute respiratory distress syndrome (ARDS) may occur, leading to pulmonary inflammation, alveolar-capillary injury, hypoxemia, and leak of proteinaceous edema with loss of lung compliance (23, 24). The main steps of AP-induced lung damage are an early marked alveolar-capillary membrane injury, due to the corrosive effect of the acid, followed by an acute, neutrophil-mediated, inflammatory response (21). Alveolar-endothelial barrier damage and neutrophil infiltration cause alterations of surfactant homeostasis and a delayed fibrotic lung tissue remodeling resulting in impaired functionality of the involved lung section.

Pulmonary surfactant is a mixture of lipids and proteins that permits normal breathing in all mammalian species forcing the alveolar liquid/air surface tension to near zero. It is synthetized by pulmonary type II cells, and it is mainly composed of phospholipids (PL), neutral lipids (cholesterol), and surfactant-specific (SP-) apoproteins (A to D). Phosphatidylcholines are the main molecular species responsible for surface tension reduction. In particular, disaturated phosphatidylcholine (DSPC) is the key component of the phospholipid film covering the alveoli due to its characteristic molecular conformation that comprises two saturated residues (mainly palmitate residues). This packed configuration avoids alveolar collapse during expiration and permits alveolar expansion during inspiration (29). Type II cells are the only cells involved in the modulation of surfactant synthesis, secretion, uptake, and recycling. Alveolar macrophages contribute to surfactant homeostasis mainly by catalyzing surfactant components (19). In acute lung injury and in lung diseases with pronounced alveolar inflammation there are significant alterations of the surfactant phospholipid profile and of the surfactant specific proteins mediated by release of cytokines and by the activated alveolar macrophages (32).

In this study we used a formerly characterized murine model of AP (1), which includes an acid load instilled only in the right bronchus, and a stable isotope approach based on the sensitivity of gas-chromatography isotope ratio mass spectrometry (GC-IRMS) to measure DSPC synthesis and secretion by means of stable isotope deuterated water, as precursor of DSPC-palmitate biosynthesis (8–9, 34). We assessed lung inflammation by protein and MPO content in both injured and noninjured lungs, as a possible compensatory mechanism due to a cross-talk between the lungs triggered by inflammation, hyperventilation, and/or undetermined type II cell reaction to the injury.

Animals with lung injury \((n = 20)\) were anesthetized prior to any procedure with a 400 mg/kg intraperitoneal injection of 2.5% Tribromoethanol (Avertin, Sigma-Aldrich, Milan, Italy). Lung injury was induced by instillation of 1.5 ml/kg HCl 0.1 M with a PE10 tube into the right bronchus through a small tracheal incision. The bronchial catheter was removed and the tracheal incision sutured. During instillation and for the next 10 min, mice were mechanically ventilated (Inspira avs, Harvard Apparatus, Holliston, MA, with the following parameters: \(V_t = \text{8} - \text{10 ml/kg; RR = 130, PEEP = 2 cmH}_2\text{O, FO}_{2} = 1\) and kept in a reverse Trendelenburg position (45°), tilted to the right side (45°) to confine the instilled fluid to the right lung. Treated animals were then placed in an oxygenated chamber (FiO\(_2\) = 0.5) until fully awake (1). We used eight healthy mice as naïve controls as they did not receive any instillation into the main right bronchus, but they were ventilated and treated as the HCl instilled mice. Five mice only received “vehicle” (NaCl 0.9%) into the right main bronchus and they were killed 24 h after the instillation, as previous studies showed that this was the peak time of lung injury (8, 39). Therefore the study included six groups of lungs: (1) injured (I) right lung; (2) contralateral noninjured (NI) left lung; (3) naïve control right lung; (4) naïve control left lung; (5) NaCl control right lung; (6) NaCl control left lung.

**Isotope infusion protocol.** We administered deuterated water 18 h after HCl instillation. Study mice (naïve controls and HCl treated mice, \(n = 4\) per control or experimental group) received an intraperitoneal dose of 1 ml/kg 10% (v/v) of deuterated water (Cambridge Isotope Laboratories, Andover, MA) to assess DSPC-palmitate synthesis and secretion. Animals were killed at 4, 8, 10, 12, 20, and 24 h after isotope administration, corresponding to 22, 26, 28, 30, 38, and 42 h after HCl injury. The age matched naïve control animals received the intraperitoneal dose of deuterated water and were killed at 4, 8, 10, 12, 20, and 24 h after isotopes administration. NaCl treated mice received no isotopes and were killed 24 h after instillation.

**Sample collection.** After opening the chest, right and left lung were subjected to selective broncho-alveolar lavage fluid (BALF) performed as follows: 0.6 ml of 0.9% NaCl was instilled in the right main bronchus three times, the withdrawal fluid was recovered and pooled in a single aliquot; 0.4 ml of 0.9% NaCl was instilled three times in the left main bronchus to obtain the left BALF (28). During the instillation procedure each lung was isolated with a clamp onto the opposite main bronchus to separate one lung during the lavage of the contralateral one. BALF was centrifuged 10 min at 1,500 \(g\) for 10 min, mice were killed at 4, 8, 10, 12, 20, and 24 h after instillation and the right BALF were collected and the three lavages of the right and left lungs of four healthy mice that were subjected to selective broncho-alveolar lavage fluid (BALF) per-
it represents the amount of DSPC synthesized in 1 day in that pool, expressed as μmol/g wet weight/day. Since DSPC is synthesized in the lung tissue and secreted into the alveolar space, for the purpose of this study we considered as indicator of DSPC synthesis, the ASR calculated in the lung homogenate, and as indicator of DSPC's secretion the ASR value calculated in the BALF. From these measurements we derived the lung DSPC turnover time (which represents DSPC pool renewed in the lungs over 24 h) using the following formula mean ASR (I/NI lungs)/sum of DSPC pool sizes (I/NI lungs).

Statistical analysis. Data were expressed as mean ± SD. Statistical power was assessed a priori by Meade’s resource equation. Comparisons between two groups were performed by Student t-test. Two-way analysis of variance (ANOVA) was used to evaluate the effects of HCl injury, time, and their interaction. One-way ANOVA with the Dunnett’s multiple comparison test was used to compare I and NI lungs with the respective control lungs at each time point. P values of less than 0.05 were considered statistically significant. Analysis was performed using Prism 4.0 (GraphPad Software, La Jolla, CA).

RESULTS

Animal protocol. Selectivity of the injury was assessed by lung macroscopic examination during the autopsy. Animals with bilateral or left injury were excluded. We previously confirmed that in this model, on a macroscopic view, the majority of the histopathologic changes were confined in the right caudal pulmonary lobe (1). In addition, to verify the selectivity of acid instillation and to exclude a relevant contamination of the left lung, in another experiment (unpublished data, under review) mice were treated with a bolus of 1.5 ml/kg of methylene blue administered in the same way used for HCl instillation and mechanically ventilated on a reverse Trendelenburg position (45°), tilted to the right side. We macroscopically confirmed a confinement of the instilled fluid in the right lung (mainly in the caudal lobe) while the parenchyma, the hilum, and the main right bronchus showed no trace of the blue dye (data not shown).

Estimate of BALF recovery from the lungs. Three sequential BALFs from four different mice were recovered and analyzed separately for both the right and the left lung. The asymptote of the resulting curve was calculated with the sample equation y = A*exp (Bx) + C (Fig. 1). We estimated a 100% recovery in 0.233 mg of DSPC from the right lungs and of 0.220 mg of DSPC mg from the left lungs. The mean DSPC amount in our (pooled) right BALF was 0.189 ± 0.05 mg, corresponding to 81% recovery of total DSPC after three lavages. The mean DSPC pool size for the left lung was 0.176 ± 0.04 mg, corresponding to an 80% recovery.

Total proteins and MPO activity. We compared the effect of NaCl and HCl on BALF protein content at 24 h after the instillation. NaCl increases BALF proteins threefold in the right lung, while HCl induced a protein leak in the right BALF of about 20-fold compared with naïve controls (naïve controls 0.62 ± 0.37; NaCl treated 2.05 ± 1.23; HCl treated 19.13 ± 7.66; measures in mg protein/g wet weight). Total protein

---

**Fig. 1.** Asymptotic calculation of bronchoalveolar lavage fluid (BALF) lavage disaturated phosphatidylcholine (DSPC) recovery. Three serial BALF were collected from the right and left lungs of four control mice and analyzed separately. DSPC amounts were plotted against the BALF serial number and the total amount of DSPC extrapolated from an exponential curve fitting. The figure shows the representative plot of the right lung experiment.

**Fig. 2.** Determination of total protein content in BALF (A) and lung homogenate (B) of injured (I) lungs compared with the respective not injured (NI) lungs. Time points 22, 26, 28, 30, 38, 42 h from acid instillation. Data are expressed as mean ± SD, n = 4 mice/time point. *P < 0.05 compared with naïve controls (Dunnett’s multiple comparison test).
content in the BALF and lung homogenate (right vs. left) were not affected by NaCl instillation ($P = 0.68$ and $P = 0.55$, respectively, by Student’s $t$-test).

Total protein content of BALF HCl I and NI lungs was significantly increased by HCl injury ($P = 0.002$) and by the time from the study start ($P = 0.002$) (both lungs, two-way ANOVA, Fig. 2a). In the lung homogenate the total protein content was only affected by HCl injury ($P < 0.0001$) (Fig. 2b).

BALF MPO activity was significantly increased in both HCl I and NI lungs over time ($P = 0.0004$, two-way ANOVA vs. naïve controls) and tended to be more pronounced in the I lungs compared with the NI lungs (I = $45.61 \pm 17.9$ mU/g wet weight, NI= $34.82 \pm 9.4$ mU/g wet weight, $P = 0.07$ by Student’s $t$-test). MPO activity in BALF was significantly increased at t26 and t28 in the I lungs and at t26 in the NI lungs during the autopsy, and animals with bilateral or left injury were excluded. In almost all mice the injury was confined in the lower lobe bronchus, confirming the accuracy of the model.

According to our previous study (1) and to current data, naïve control mice and NaCl 0.9% (vehicle) treated mice did not differ from their respective naïve control lungs.

**DISCUSSION**

In this study we used body water-derived deuterium incorporation into lipids to trace the synthesis and secretion of the most important subfamily of surfactant phospholipids, DSPCs, in a previously characterized model of unilateral acid-induced lung injury (1). The aim of the study was to assess if a localized injury influences surfactant regulation only in the afflicted (confined) lung space or if a local damage could affect the whole pulmonary surfactant system. In this model, selectivity of the injury was assessed by lung macroscopic examination during the autopsy, and animals with bilateral or left injury were excluded. In almost all mice the injury was confined in the lower lobe bronchus, confirming the accuracy of the model.

According to our previous study (1) and to current data, naïve control mice and NaCl 0.9% (vehicle) treated mice did not differ from their respective naïve control lungs.

**DISC**

**P**

**SD**

**DISCUSSION**

In this study we used body water-derived deuterium incorporation into lipids to trace the synthesis and secretion of the most important subfamily of surfactant phospholipids, DSPCs, in a previously characterized model of unilateral acid-induced lung injury (1). The aim of the study was to assess if a localized injury influences surfactant regulation only in the afflicted (confined) lung space or if a local damage could affect the whole pulmonary surfactant system. In this model, selectivity of the injury was assessed by lung macroscopic examination during the autopsy, and animals with bilateral or left injury were excluded. In almost all mice the injury was confined in the lower lobe bronchus, confirming the accuracy of the model.

According to our previous study (1) and to current data, naïve control mice and NaCl 0.9% (vehicle) treated mice did not differ from their respective naïve control lungs.

Table 1. Kinetic parameters and mean pool size of bronchoalveolar lavage fluid and lung homogenates from HCl injured and naïve control mice

<table>
<thead>
<tr>
<th></th>
<th>Right (Injured) BALF</th>
<th>Left (Contralateral) BALF</th>
<th>Right (Injured) Tissue</th>
<th>Left (Contralateral) Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive controls HCl model (I)</td>
<td>16.3</td>
<td>23.0</td>
<td>13.1</td>
<td>26.7</td>
</tr>
<tr>
<td>Naive controls HCl model (NI)</td>
<td>26.7</td>
<td>30.9</td>
<td>29.2</td>
<td>30.9</td>
</tr>
<tr>
<td>DSPC-PA FSR, % synthesis/day</td>
<td>36.0</td>
<td>30.9</td>
<td>30.9</td>
<td>30.9</td>
</tr>
<tr>
<td>DSPC-PA ASR, μmol/g wet weight/day</td>
<td>2.2 ± 0.7</td>
<td>1.8 ± 0.3</td>
<td>2.1 ± 0.6</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>DSPC pool size, μmol/g wet weight</td>
<td>2.1 ± 0.6</td>
<td>1.7 ± 0.5</td>
<td>1.3 ± 0.4</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

Eighteen hours after injury, mice received an intraperitoneal dose of deuterated water 10% vol/vol, 1 ml/kg. Samples were collected at 4, 8, 10, 12, 20, 24 h after isotope dose, that corresponded to 22, 26, 28, 30, 38, 42 h after acid instillation. Fractional synthesis rate (FSR) was calculated using the slope of the enrichment versus time curves of surfactant disaturated phosphatidylcholine (DSPC)-palmitate. Absolute synthetic rate (ASR) was calculated by multiplying FSR by DSPC pool size. BALF = bronchoalveolar lavage fluid; I = acid injured lungs; NI = contralateral, noninjured lungs; DSPC-PA = DSPC-palmitate; $n = 4$ mice/each time point.
not show a significant difference on their inflammatory status; hence we used healthy mice as naïve controls for most of the comparisons. Local lung injury and endothelial dysfunction were assessed by MPO activity and total protein content, and results were in accordance with our previous study (1).

The major findings of this study were 1) HCl injury significantly decreased BALF DSPC pool size of the I lungs compared with the NI and control lungs. 2) HCl injury significantly increased the DSPC pool of the NI lungs compared with the I and control lungs. 3) Lung tissue (homogenates) DSPC pools of I and NI lungs did not change, and they remained similar to the respective naïve controls lungs.

To further explain these findings, we used deuterated water as metabolic precursor of DSPC to measure surfactant DSPC synthesis and secretion of the I and NI lungs, together with total lungs DSPC turnover (7, 10). Below we briefly comment on the effect of DSPC metabolism on lung homogenate DSPC pools and BALF DSPC pools.

**Lung homogenate DSPC pools.** In lung homogenate DSPC-palmitate synthesis was increased by 14.3% in the I lungs and by 40.8% in the NI lungs compared with naïve control mice (Table 1), suggesting a cross-talk mechanism that affects also the lung parenchyma contralateral to the injury. In patients with unilateral pneumonia, Dehoux MS et al. (11) reported a confined inflammatory response on the site of infection and, at the same time, a systemic inflammatory response mediated by interleukin 6 (IL-6) that was also increased in the noninfected lung compartments (contralateral lung). Recent studies in mice receiving intraperitoneal lipopolysaccharide showed that STAT-3 (signal transducer and activator of transcription-3), activated by members of the IL-6 like group of proinflammatory cytokines, increased DSPC and SP-B concentration in lungs and in the alveolar space (17), leading to increased surfactant pools and improved lung functionality and recovery. Moreover, ABCA3-knockout mice showed a compensatory lipid synthesis in nontargeted type II cells, indicating that surfactant homeostasis was a highly regulated process that included sensing and coregulation among alveolar type II cells (4). In our study, tissue DSPC pools remained unchanged in both lungs compared with naïve controls; thus it looked like DSPC synthesis and secretion were regulated in such a way that tissue DSPC pool remained constant (even if the rates of synthesis and secretion were accelerated). To support this finding, it is interesting to note that Zhou J. et al. (40) reported that an overexpression of cholinephosphate cytidylyltransferase alpha, an enzyme involved in surfactant de novo synthesis, was balanced by a basolateral phospholipid efflux mediated by the ABCA1 transporter. The overall result was that even in the presence of an enhanced PC synthesis, the PC concentration remained constant inside the cell.

**BALF DSPC pools.** BALF DSPC pools were significantly diminished in I lungs compared with the NI and control lungs. In ARDS patients we recently reported that DSPC amount in tracheal aspires was markedly reduced and DSPC FSR was 3.1 times higher than in control patients (34), suggesting an overall compensatory DSPC synthetic mechanism that was still not sufficient to replace the DSPC lost during the acute phase of lung injury.

We speculate that DSPC loss from the BALF could be explained by activation of the alveolar macrophages and increased DSPC catabolism. Shanley et al. demonstrated that macrophage inflammatory protein (MIP-2) was upregulated in aspiration-induced lung injury in rats, leading to an accumulation of neutrophils and macrophages via a chemotactic mechanism (33). These cells play a critical role in alveolar surfactant catabolism and turnover (22, 20, 13). Interestingly, we found that total DSPC turnover of the HCl-treated mice was almost twofold the DSPC turnover of the naïve controls.

The NI lung DSPC secretion was 37.6% faster than in the respective naïve control lungs and 64.3% more umol/g wet lung/day of DSPC than in the I lungs, leading to an increased DSPC pool size. It seems unlikely that the increased DSPC secretion was due to a Type II cell hyperplasia observed in response to lung injury (1). Type II cells rapidly differentiate into Type I cells after lung injury, which act as an air/body barrier with little or no function in surfactant homeostasis (13, 14, 38). On the other hand, it seems more likely that the inflammatory reaction and hyperventilation, by stretching the alveoli, induced an increase of DSPC secretion and pool boost especially in the NI lung (27), where surfactant could be stored as a reservoir for both lungs. Whether surfactant phospholipids could migrate from one lung to the other is unknown. Previous studies reported that ciliary movements are probably the main factor responsible for surfactant clearance from the small to the large airways (15). In addition, Espinosa FF et al. (12) demonstrated, with a mathematical model, that a gradient of surface tension in the airways could spread the surfactant in a matter of seconds (12 s from the trachea to the alveoli). Whether this mechanism is responsible for a surfactant exchange between the lungs is still a speculation.

In summary, by combining the efficacy of a well-characterized model of lung injury and the sensibility of stable isotopes/GC-IRMS techniques, we showed that after a local injury the entire lung system is involved in responding to the damage by activating the immune system and surfactant synthesis. We speculate that surfactant catabolism and synthesis are two interdependent pathways and that it is likely that synthesis undergoes a “whole organ” regulation while inflammation/catabolism has its climax locally and it gradually decreases away from the injury.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


