Surfactant function in children with chronic airway inflammation

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Braun, A., M. Steinecker, S. Schumacher, and M. Griese. Surfactant function in children with chronic airway inflammation. J Appl Physiol 97: 2160–2165, 2004. First published August 13, 2004; doi:10.1152/japplphysiol.00523.2004.—Pulmonary surfactant is necessary to keep the terminal conducting airways patent. It is unknown whether mild to moderate airway inflammation may influence surfactant function and thus contribute to the pathogenesis of chronic airway inflammation in children. To answer this question, 21 children with chronic obstructive bronchitis and 19 asymptomatic children with long-term tracheostomy and increased numbers of neutrophils in their airways were compared with 15 healthy controls. Bronchoalveolar lavage fluid was separated into large surfactant aggregates (LA) and a supernatant containing inhibitory constituents. Surfactant function of LA, recombinations of LA and supernatant, and recombinations of a defined bovine surfactant and supernatant was assessed in a capillary surfactometer. Compared with controls, the function of the LA surfactant was reduced and there was no difference between children with tracheostomy and chronic obstructive bronchitis. The function of LA-supernatant recombinations was poor in all subjects. This may be explained by the well-known protein influx during the lavage procedure. The activity of bovine surfactant-supernatant reconstitutions was impaired in children with tracheostomy. In all surfactant mixtures assessed, surfactant function was inversely correlated to the number of neutrophils in the lavage fluid. Chronic lower airway inflammation with mild or no clinical symptoms is associated with impaired surfactant function. The dysfunction may contribute to airflow restrictions frequently observed in these children.

PULMONARY SURFACANT IS ESSENTIAL to prevent alveolar collapse and to guarantee the patency of the terminal conducting airways (1, 16, 27). Reduced surfactant activity is noticeable in various acute pulmonary diseases, such as the acute respiratory distress syndrome, asthma, and pneumonia (11). Little is known about alterations of the surfactant system in clinically stable patients with chronic airway inflammation. Most of the studies dealing with this subject were performed in older children or adults (9, 12, 32) or in animals (5, 25). No studies on young children have been published to our knowledge. In chronic airway inflammation, damage to the surfactant system is possible even if the degree of inflammation is only mild or moderate. The mechanisms known to reduce surfactant activity, e.g., surfactant inhibition by lytic enzymes (1), cellular lipids (18), and blood-derived proteins (22) leaking into the airway lumina during inflammation, may all be active in chronic bronchitis, although to different degrees.

The ability of surfactant to keep small airways open can be investigated with a capillary surfactometer. This instrument has been developed to assess surfactant function in airway diseases (8, 26). Small amounts of material are necessary for each measurement. With the use of this method in adult patients with mild allergic asthma, it was recently shown that the pulmonary surfactant was impaired in its ability to maintain airway patency after allergen challenge (17, 20). For our purpose, i.e., to assess the biophysical function of the surfactant system in young children with stable airway inflammation, the capillary surfactometer appeared to be the most suitable tool.

We asked the question whether mild to moderate inflammation in children with chronic bronchitis, particularly in its obstructive form, may have an impact on surfactant function and thus may be an important contributing pathogenic factor to restrict airflow in the distal conducting airways. Therefore, children with mild symptoms of chronic obstructive bronchitis and another group of asymptomatic children, but with long-term tracheostomy and associated inflammation, were compared with control children with respect to airway inflammation and surfactant function.

METHODS

Demographic and clinical data as well as bronchoalveolar lavage (BAL) findings of the study subjects are shown in Table 1. In all children, informed consent was obtained from the parents before BAL. The study was approved by the ethics committee.

Children with chronic obstructive bronchitis. We investigated 21 children (14 male individuals) with a median age of 5.3 (2.7–7.5) yr. Lavages were performed to exclude anatomic abnormalities and to assess the activity of airway inflammation. We included children with chronic coughing and wheezing (>3-mo duration) diagnosed as chronic obstructive bronchitis by their attending physician or a clinic pediatrician. Subjects in whom an obvious reason for chronic inflammation was detected with bronchoscopy, such as stenosis or chronic foreign body aspiration, were excluded. At the time of lavage, none of the subjects suffered from an acute exacerbation of symptoms. Caregivers were contacted per telephone 27 (17–35.5) mo after the examination and asked about the clinical course and medication after BAL. The parents of 13 subjects reported diminished coughing and wheezing, in four children symptoms had not clearly changed, and only one child was reported to suffer from an increased disease activity. Three children could not be evaluated with the questionnaire.

Children with long-term tracheostomy. Nineteen patients (10 male individuals) with long-term tracheostomy (>3 mo), being treated as outpatients, were studied. They had no clinical symptoms. The median age was 3.9 (2.6–5.6) yr. These subjects belong to a population of children with tracheostomy who had undergone lavage for the purpose of a prospective trial to assess airway inflammation and biochemical surfactant alterations (14). Four patients were in need of tracheostomy because of an airway obstruction located proximally of the larynx, 12 because of intralaryngeal, and 3 because of tracheal obstruction. Children with congenital metabolic disorders such as mucopolysaccharidosis and with underlying pulmonary diseases were excluded.

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Table 1. Clinical, cellular, and biochemical characteristics of the subjects

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 15)</th>
<th>Obstructive Bronchitis (n = 21)</th>
<th>Tracheostomy (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>9/6</td>
<td>14/7</td>
<td>10/9</td>
</tr>
<tr>
<td>Age, yr</td>
<td>4.7 (3.9–4.7)</td>
<td>5.3 (2.7–7.5)</td>
<td>3.9 (2.6–5.6)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>18.5 (13.6–26.7)</td>
<td>14 (11.5–19)</td>
<td>20.7 (15.1–29.4)</td>
</tr>
<tr>
<td>History of atopy or allergy, no.</td>
<td>3</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Inhaled steroids at time of BAL, no.</td>
<td>0</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Recovery, %</td>
<td>64</td>
<td>59</td>
<td>64</td>
</tr>
<tr>
<td>Total cell count, ×10⁶ cells/ml</td>
<td>11.5 (8.1–17.5)</td>
<td>15 (9.3–27.3)</td>
<td>20 (14–36.5)*</td>
</tr>
<tr>
<td>Macrophages, ×10⁶ cells/ml</td>
<td>9.9 (6.7–15.2)</td>
<td>14.1 (7.1–19.5)</td>
<td>12.5 (8.5–22.7)</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>84.5 (79–89.5)</td>
<td>79 (67.5–89)</td>
<td>79 (67.5–89)</td>
</tr>
<tr>
<td>Lymphocytes, ×10⁶ cells/ml</td>
<td>1.6 (1.0–2.3)</td>
<td>1.9 (0.8–3.0)</td>
<td>1.9 (0.8–3)</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>13 (9.0–19.9)</td>
<td>10 (5–16)</td>
<td>12 (6–22)</td>
</tr>
<tr>
<td>Neutrophils, ×10⁶ cells/ml</td>
<td>0.2 (0.1–0.4)</td>
<td>0.5 (0.2–2.2)</td>
<td>1.1 (0.4–9.4)†</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>1.5 (1–3)</td>
<td>2 (1.5–10.5)</td>
<td>4 (2–45.9)*</td>
</tr>
<tr>
<td>Eosinophils, ×10⁴ cells/ml</td>
<td>0 (0–0.1)</td>
<td>0 (0–0.3)</td>
<td>0.1 (0–0.8)</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>0 (0–0.6)</td>
<td>0 (0–0.2)</td>
<td>0.8 (0–2)</td>
</tr>
<tr>
<td>Total phospholipid in LA fraction, µg/ml BAL fluid</td>
<td>15.1 (9.2–35.9)</td>
<td>24.4 (14.8–38)</td>
<td>21.9 (18.1–66.9)</td>
</tr>
<tr>
<td>Protein (SA)-to-PL (LA) ratio</td>
<td>2.3 (1.9–5.3)</td>
<td>2.7 (2.1–5)</td>
<td>2.7 (1.7–5.6)</td>
</tr>
</tbody>
</table>

Values are medians with interquartile ranges; n, no. of subjects; LA, large surfactant aggregate; SA, small surfactant aggregate; PL, phospholipid; M, male; F, female; BAL, bronchoalveolar lavage. Comparisons between the 3 groups were done by Kruskal-Wallis test and Dunn’s post hoc test. *Significant difference, P < 0.05. †Significant difference, P < 0.01.

None of the children suffered from acute lower respiratory tract symptoms at the time of BAL.

Controls. Controls were 15 children (9 male individuals; median age 4.7 yr; 3.9–9.9 yr) undergoing elective surgery for nonpulmonary diseases such as adenoeotmy, tonsillecory, ganglion extirpation, or circumcision. None of the children suffered from an acute respiratory tract infection within the preceding 4 wk. No subject had a history of chronic lower respiratory tract symptoms. These children were from the same cohort as the tracheostoma patients, as described previously in detail (14).

Evaluation of biophysical surfactant function. The evaluation of surfactant function was performed with a capillary surfactometer (Calmia Medical, Toronto, Ontario, Canada). This device has been constructed to test the ability of surfactant to keep the smallest conducting airways patent during respiration (26). Briefly, it works with a glass capillary having a short narrow section with an inner diameter of 0.25 mm to simulate human terminal bronchioles (19). To assess the function of a sample fluid, 0.5 µl is deposited in this section. The narrow section of the capillary is thus blocked by a column of liquid that will be extruded when pressure is raised at one end of the capillary. If the liquid contains well-functioning surfactant, it will stay in the wide section of the capillary and will not return to the narrow part. The capillary is then open for a free airflow so that pressure remains at zero because it does not meet resistance. If the sample contains surfactant with a lack of quality or quantity, the liquid column will flow back to the narrow section of the capillary several times within the 120-s period. This will be due to its high surface tension. The capillary surfactometer calculates the proportion of time during which the capillary was patent, related to the total measurement time of 120 s. The result is expressed as “% open” and indicates biophysical surfactant function of a sample; “100% open” corresponds to optimally functioning pulmonary surfactant. All measurements with the capillary surfactometer were performed at least in triplicate. For each measurement, a new glass capillary (Calmia Medical) was used.

BAL fluid. BAL was performed under general inhalational anesthesia with O₂, N₂O, and 1–5% halothane. A fiber-optic pediatric 3.5-mm endoscope was used for bronchoscopy and was wedged mainly in the right inferior lobe or one of its segments and in few cases in the right superior or middle lobe. For BAL, four aliquots of 1 ml prewarmed isotonic saline/kg body weight were instilled and immediately withdrawn by gentle suction. Fractions two to four were pooled, and an aliquot was used for semiquantitative microbiological examination. BAL fluid was filtered through gaze to remove mucus and centrifuged at 200 g for 10 min to pellet the cells. Cytospin preparations were stained with May-Grünwald-Giemsa. The supernatant was stored at −80°C.

BAL fluid preparation and analysis. The pooled cell-free supernatant was centrifuged for 30 min at 40,000 g at 4°C to obtain a lipid pellet consisting of “large surfactant aggregates” (LA) and a supernatant containing “small surfactant aggregates” (SA). The lipid pellets were resuspended in distilled water (1 ml each). Lipid extraction of the suspensions was performed according to Bligh and Dyer (2). The phospholipid concentration was determined from the inorganic phosphate contents of the lipid extracts (7). The supernatants of high-speed centrifugation (SA) were desalted (9 h, 7,500 g, 4°C) in 1-kDa microconcentrators (Pall Filtron Technology, Northborough, MA). Total protein contents of the concentrated SA fractions were determined according to Bradford (4). All phospholipid and protein measurements were done in duplicate.

We evaluated the function of LA suspensions, recombinations of SA with LA, and recombinations of SA with well-functioning native bovine surfactant with the capillary surfactometer. For the LA suspensions, volumes of the native LA suspensions, corresponding to 10 µg of phospholipids, were prepared. To conserve the ratio of supernatant (SA) to the LA fraction, which was present within native BAL fluid samples, recombinations of SA and LA for determination of surfactant function were prepared as follows. A volume of the respective LA suspension containing 10 µg of total phospholipids each was multiplied with the volume of native BAL fluid utilized for high-speed centrifugation (ml), divided by 1 ml. This was necessary because the LA fraction had been resuspended in 1 ml of distilled water for all patients and controls. The resulting volume was pipetted from SA supernatants and concentrated by microconcentration (cutoff <1 kDa) before being added to the LA fraction (10 µg). To quantify the inhibitory potential of SA fractions, which contain water-soluble proteins, e.g., from plasma, defined volumes of the supernatant (SA) from patients and controls were added to well-functioning native bovine surfactant. The volumes of supernatant used were based on defined amount of total protein. A mass ratio of 2:1 (20 µg of total protein in the supernatant to 10 µg of total phospholipid in bovine surfactant) was reconstituted within the samples to be measured. This ratio was chosen with regard to experiments with supernatants from several patients at various dilutions. They showed that a ratio of...
Comparison of surfactant function between the three groups.
The function of the LA fraction isolated from the three groups of subjects was significantly different (overall $P = 0.0002$; Fig. 1). Both groups with chronic airway inflammation had worse % open values than the control subjects ($P < 0.001$ for both). In both the obstructive bronchitis group and the tracheostomy group, % open results ranged almost from 0 to 100%. In contrast, none of the controls had a % open value lower than 50% (Fig. 1). Reconstituting the SA with the LA fractions reduced surfactant function to an extremely poor surfactant ability to maintain capillary patency in all patients and controls. Results were similar in both groups with respiratory tract inflammation and control subjects (Fig. 2).

To determine potential differences between the inhibitory capacity of defined amounts of the SA fractions, these were added to well-functioning bovine surfactant at a SA-protein to LA-phospholipid ratio of 2:1 (by weight). In all groups, the surfactant activity was reduced compared with bovine surfactant alone (Fig. 3). There was no significant difference between the three groups of study subjects.

Correlations between cytological, microbiological, and clinical parameters and surfactant function. A consistent and negative correlation was found between surfactant function and inflammatory markers, i.e., absolute and relative numbers of neutrophils in BAL fluid. This was observed for the LA suspensions, the SA/LA recombinations and the SA/bovine surfactant mixtures (Table 2). Interestingly, surfactant function did not depend on the clinical course reported by the caregivers of the children with chronic obstructive bronchitis 27 mo after BAL or the application of inhaled steroids at the time of BAL (data not shown), nor did surfactant function depend on bacterial colonization (data not shown).

DISCUSSION
In this study, we confirmed our hypothesis by more evidence that alterations to the pulmonary surfactant system might play a pathogenic role not only in acute but also in chronic and clinically stable airway inflammation. Our results showed a significantly reduced function of the LA fractions from the lavages of children with inflammation in relation to chronic tracheostoma or symptomatic chronic obstructive bronchitis.
In the tracheotomized children, BAL fluid neutrophil numbers were elevated compared with the controls. This indicates a bronchopulmonary inflammatory reaction despite the lack of clinical symptoms. Tracheotomized children are predisposed toward airway inflammation as a consequence of the bypass of the protective upper respiratory tract. Thus they are to a high degree exposed to microorganisms, which is underlined by the increased frequency of positive bacterial culture in 65% compared with 36% in the control group. In contrast, children with obstructive bronchitis and airway symptoms yielded reduced LA function, although the number and percentage of BAL fluid inflammatory cells did not differ significantly from the controls.

All results obtained in this and similar ex vivo studies must be interpreted cautiously, because the exact in vivo conditions within the lining fluid of the respiratory tract are not known (21). We used conditions comparable to those of others (6, 10) and to our laboratory’s previous studies (11, 15). All samples were investigated at pH 6.9, 3.5 mM CaCl₂, and the NaCl concentration resulting from the salt solution used for BAL, i.e., of ~140 mM. The phospholipid concentration was adjusted to 1 mg/ml in all subjects to allow a valid comparison of the surfactant quality between various subjects, excluding dilutional effects by BAL and differences in individual surfactant concentration.

**LA function.** The LA fraction of the surfactant isolated from the lavages is principally responsible for the main part of biophysical surfactant activity (13, 29). The ability of LA to maintain airway patency was significantly reduced in children with chronic airway inflammation compared with controls. Because the phospholipid content was standardized in all samples, the quality of the surfactant must have been reduced in the two disease groups. However, there was individual variation because % open values scattered over a broad range in both patient groups, whereas all the controls had good or excellent surfactant activity. Thus in some children with mild chronic inflammation of the lower respiratory tract, surfactant function was impaired and may contribute to their obstruction of peripheral airways.

Our study was not designed to investigate the biochemical causes of the surfactant dysfunction. However, because the LA fraction contains only small amounts of water-soluble inhibitors, we suggest that its dysfunction was generated by changes in the biochemical profile. Additional washing might have removed potential water-soluble inhibitors from the LA fraction. However, this could not be done because of very limited sample material available, resulting under these conditions in variable and reduced recovery of LA (unpublished observation). Previous publications have shown changes of surfactant composition, such as decreased concentration of the major surface active component dipalmitoylphosphatidylcholine or elevated sphingomyelin levels in pneumonia, cystic fibrosis, acute respiratory distress syndrome, and other pulmonary diseases (11, 16, 28). Furthermore, a loss of surfactant function may be caused by an increased release of phospholipase A₂ or other lysolipases or phospholipases from inflammatory cells during airway inflammation (1). This might lead to hydrolysis and loss of function of dipalmitoylphosphatidylcholine, which is the principal surface active component in pulmonary surfactant.

**Function of SA-LA reconstitutions.** The SA fractions of the surfactant recovered by BAL contain the majority of surfactant-inhibiting components (12, 17). As expected, the surfactant activity was distinctly deteriorated in SA-LA reconstitutions compared with LA alone. Surprisingly, there were no differences between disease and control groups, and surfactant

### Table 2. Analysis of the correlation between surfactant function of various surfactant fractions and the recovered BAL cells

<table>
<thead>
<tr>
<th>Surfactant Function of</th>
<th>Large aggregates</th>
<th>Large and small aggregate reconstitutions</th>
<th>Small aggregate and bovine surfactant reconstitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( r )</td>
<td>( P )</td>
<td>( n )</td>
</tr>
<tr>
<td>Total number of cells</td>
<td>-0.37</td>
<td>0.006*</td>
<td>54</td>
</tr>
<tr>
<td>Number of macrophages</td>
<td>-0.15</td>
<td>0.27</td>
<td>54</td>
</tr>
<tr>
<td>Proportion of macrophages</td>
<td>0.33</td>
<td>0.01*</td>
<td>54</td>
</tr>
<tr>
<td>Number of lymphocytes</td>
<td>-0.21</td>
<td>0.14</td>
<td>54</td>
</tr>
<tr>
<td>Proportion of lymphocytes</td>
<td>0.07</td>
<td>0.6</td>
<td>54</td>
</tr>
<tr>
<td>Number of neutrophils</td>
<td>-0.45</td>
<td>0.0006†</td>
<td>54</td>
</tr>
<tr>
<td>Proportion of neutrophils</td>
<td>-0.49</td>
<td>0.0002†</td>
<td>54</td>
</tr>
<tr>
<td>Number of eosinophils</td>
<td>-0.16</td>
<td>0.24</td>
<td>54</td>
</tr>
<tr>
<td>Proportion of eosinophils</td>
<td>-0.13</td>
<td>0.35</td>
<td>54</td>
</tr>
</tbody>
</table>

Results from all subjects studied were combined, and correlations were assessed by Spearman’s rank correlation analysis. Surfactant function of the various fractions or preparations was assessed in a capillary surfactometer, and results are expressed as “% open.” \( n \), No. of subjects studied. *\( P < 0.05 \). †\( P < 0.005 \).
function was deficient in all subjects. At first glance, this contradicts data recently obtained with the capillary surfactometer, indicating relatively good % open values for SA-LA recombinations in healthy and asthmatic adults (32). However, these results cannot be compared with ours, because the SA-protein-to-LA-phospholipid ratio used for reconstitution was ~10 times lower than in this study.

To approach in vivo conditions, we reconstituted SA and LA fractions in their relative proportions as retrieved by lavage. This may not truly reflect the in vivo situation, because the local surfactant system may be disturbed by the BAL procedure. Even in healthy subjects, it has previously been demonstrated that there was some leakage of serum proteins from the vascular compartment into the air spaces, as shown in studies with radiolabeled albumin (30, 31). Inhibitory substances, which were originally located in airways proximal to terminal bronchioli, may also have been mixed with other, more peripherally located components, causing the ex vivo surfactant dysfunction. With the use of the sensitive system of the capillary surfactometer to assess surfactant function, our data clearly suggest that the complete material recovered by BAL does not make up the in vivo surfactant system, pointing out the principal limitation of all studies using surfactant recovered by BAL procedures.

Function of SA-bovine surfactant recombinations. To investigate the properties of the inhibitory SA fraction in more detail, the modulation of a well functioning bovine surfactant was assessed. When a fixed phospholipid concentration was used, the % open values declined exponentially with increasing amounts of serum or SA from the patients added. Consequently, it is only within a narrow window of protein-to-phospholipid-ratios (from ~0:1 to 1:5:1 by weight for serum-surfactant mixtures) that % open values may vary over a wide range, before falling to nearly 0%. Small quantitative increases in SA inhibitor concentrations with various disease states preclude to detect potential differences between individuals, because all subjects will have very low values, even when an excellent LA is present. In addition, qualitative differences between the three groups of subjects were suggested from diminished % open values in the tracheotomized patients compared with the controls.

Surfactant dysfunction in peripheral airway inflammation may result from a less tight barrier between airway lumina and the vascular compartment, allowing surfactant inhibitors, which are mainly serum proteins, to leak into the airways (24). Our data suggest that total protein leakage is not increased during chronic, but mild, airway inflammation compared with healthy children. However, a somewhat increased inhibitory capacity of the SA fractions from the patients may be the result of changes in the composition or inhibitory potentials of the leaked proteins. Selective damage of the epithelial barrier within the different disease states or differential expression of protein species with strong or weak inhibitory potential may contribute to surfactant dysfunction in chronic inflammation.

Correlation analysis. Impaired surfactant function clearly correlated with the degree of neutrophilic airway inflammation. These data strongly support a role of chronic airway inflammation for surfactant dysfunction. Both water-soluble (SA) and hydrophobic (LA) surfactant components are involved. Among other mechanisms, the mixing of cellular debris with surfactant lipids, the inactivation of surfactant-associated proteins by neutrophil elastase, and oxidative damage may account for LA surfactant dysfunction, especially in those subjects with increased numbers of neutrophils (3, 23, 28).

In conclusion, our data show that surfactant function was impaired in chronic airway inflammation, even in children with mild or no clinical symptoms. The presence of neutrophils may play a central role in a multifactorial process, resulting in surfactant dysfunction, which itself may contribute to peripheral airways disease in children with chronic obstructive bronchitis or long-term tracheostomy.

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REFERENCES


