Lung surfactant in a cystic fibrosis animal model: increased alveolar phospholipid pool size without altered composition and surface tension function in \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice

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Abstract

\textbf{Background} – Progressive pulmonary dysfunction is a characteristic symptom of cystic fibrosis (CF) and is associated with functional impairment and biochemical alterations of surfactant phospholipids in the airways. However, the fundamental question of whether surfactant alterations in the CF lung are secondary to the pulmonary damage or are present before initiation of chronic infection and inflammation has yet to be resolved in patients with cystic fibrosis but can now be addressed in CF mice that exhibit the basic defect in the airways. A study was therefore undertaken to investigate the pool sizes, composition, and function of lung surfactant in the non-infected \textit{cftr}\textsuperscript{m1HGU/m1HGU} mouse.

\textbf{Methods} – The amount and composition of phospholipid classes and phosphatidylcholine molecular species were determined in bronchoalveolar lavage (BAL) fluid and lavaged lungs by high performance liquid chromatography (HPLC). Surfactant protein A (SP-A) in BAL fluid were determined by ELISA and surfactant for functional measurements was isolated from BAL fluid by differential ultracentrifugation. Equilibrium and minimal surface tension of surfactant was assessed by the pulsating bubble surfactometer technique. MF1, BALB/c, C57BL/6, and C3H/He mice served as controls.

\textbf{Results} – BAL fluid of \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice contained 1.02 (95% confidence interval (CI) 0.89 to 1.16) μmol phospholipid and 259 (239 to 279) ng SP-A. BAL fluid of MF1, BALB/c, C57BL/6, and C3H/He mice contained 0.69 (0.63 to 0.75), 0.50 (0.42 to 0.57), 0.52 (0.40 to 0.64), and 0.45 (0.27 to 0.63) μmol phospholipid, respectively. After correction for the different body weights of mouse strains, phospholipid levels in BAL fluid of \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice were increased by 64 (52 to 76)%, 60 (39 to 89)%, 72 (45 to 113)%, and 92 (49 to 163)%, respectively, compared with controls. The amount of SP-A in BAL fluid and the composition of phospholipid as well as phosphatidylcholine molecular species in BAL fluid and lung tissue was unchanged in \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice compared with controls. The increase in phospholipids in BAL fluid of \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice resulted from an increased fraction of large aggregates which exhibited normal surface tension function.

\textbf{Conclusion} – In \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice surfactant homeostasis is perturbed by an increased phospholipid pool in the alveolar compartment.

Keywords: cystic fibrosis, surfactant pool size, \textit{cftr}\textsuperscript{m1HGU/m1HGU} mouse.

Cystic fibrosis is an inherited disease with a defect in the cystic fibrosis transmembrane conductance regulator (\textit{CFTR}) gene. This gene codes for an integrated plasma membrane protein which is responsible for cAMP regulated chloride transport through the plasma membrane.\textsuperscript{12} Mucus viscosity is increased in bronchial secretions from patients with cystic fibrosis.\textsuperscript{13} Additionally, airway surfactant from patients with cystic fibrosis chronically infected with \textit{Pseudomonas aeruginosa} shows impaired function and altered phospholipid composition, possibly due to enzymatic destruction and contamination of surfactant secondary to infection.\textsuperscript{5} However, a primary alteration of surfactant metabolism in cystic fibrosis may additionally exist since the \textit{CFTR} gene is expressed in Clara cells and type II pneumocytes.\textsuperscript{67} Type II pneumocytes synthesise and secrete all the phospholipid and apoprotein components of surfactant, while Clara cells release surfactant apoproteins SP-A, SP-B, and SP-D into the airways.\textsuperscript{89} Surfactant phospholipid comprises about 80% phosphatidylcholine (PC), with dipalmitoyl phosphatidylcholine (PC16:0/16:0) being the most important molecular species, and about 10% phosphatidylglycerol (PG). Phospholipid homeostasis may be generally altered in cystic fibrosis since the rate of choline incorporation into PC is increased in isolated blood platelets and fibroblasts from patients with cystic fibrosis.\textsuperscript{10} It is possible that defective \textit{CFTR} gene expression may alter surfactant phospholipid homeostasis before colonisation of the lungs with opportunistic bacteria. Since the concentration of surfactant phospholipids and the ratio between individual components, particularly between SP-A and phospholipids, can influence the anti-
body-independent defence functions of surfactant,\textsuperscript{11} altered phospholipid homeostasis may consequently affect pulmonary integrity. To investigate any such primary alterations in surfactant homeostasis we analysed the concentration of surfactant phospholipids and SP-A in lungs of \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice.\textsuperscript{12,13} We further investigated in this mouse model the composition of surfactant phospholipid classes as well as phosphatidylycholine molecular species and, finally, the surface tension function of lung surfactant in comparison with healthy controls. In \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice a \textit{cftr} gene defect was generated by insertional mutagenesis into exon 10 of the \textit{cftr} gene\textsuperscript{12} in which aberrant skipping results in the synthesis of 5–10% wild type transcript and therefore relatively mild symptoms of \textit{cftr} deficiency.\textsuperscript{13} It was our aim to determine whether there was any primary deviation in pulmonary phospholipids of \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice in the absence of chronic pulmonary infection.\textsuperscript{5}

**Methods**

**MATERIALS**

Specific pathogen-free \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice supplied from Edinburgh\textsuperscript{12} as well as Ztm:MF1, BALB/cZtm, C57/BL6Ztm, and C3H/HeZtm mice were bred at our local animal house. \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice originated from five different breeding lines. Mice were kept in a flexible film isolator under specified pathogen-free conditions. The hygienic status was tested monthly according to Kunstyr\textsuperscript{14} and the mice were free from murine pathogens. The temperature within the isolator was maintained at 20–24°C with 40–50% relative humidity. Animals were fed an irradiated (5 Mrad) standard diet (Altromin 1314) and autoclaved water (134°C, 50 minutes) was given ad libitum. Mice subjected to analysis were 5–7 weeks old and the numbers of experiments were not based on any pre-study power calculations. High performance liquid chromatography (HPLC) grade solvents were supplied by Baker (Deventer, Holland). All other solvents and chemicals were of analytical grade and from various commercial sources.

**HISTOLOGICAL EXAMINATION**

For histological examination \textit{cftr}\textsuperscript{m1HGU/m1HGU} mice (\textit{n} = 6, 6–10 weeks old) and MF1 mice (\textit{n} = 6) were sacrificed by cervical dislocation and bled by incision of the abdominal aorta. The thorax was opened and, after perfusing the lung with 1 ml cold phosphate buffered saline via the right ventricle, the trachea was cannulated with a small catheter. The lung was then filled with 1 ml OCT embedding medium (Miles Inc, Elkhart, Indiana, USA) diluted 1:4 with 4% buffered formalin and further immersed in 4% buffered formalin for fixation. Paraffin-embedded sections (5 μm) were prepared and stained with haematoxylin and eosin.

**PHOSPHOLIPID EXTRACTION**

For phospholipid quantitation in cell-free BAL fluid two aliquots of 300 μl were placed into phosphate-free glass tubes, the volumes adjusted to 1 ml with 154 mM saline, and this material was extracted with chloroform/methanol according to the method of Bligh and Dyer.\textsuperscript{15} The chloroform extract was dried under a stream of nitrogen and the glass tube directly used for phospholipid quantitation. To quantify phospholipids in subfractions of BAL fluid aliquots of P60000 and S60000 were extracted with chloroform/methanol as outlined above. Lavaged lungs were extracted according to Folch et al.,\textsuperscript{16} the extract adjusted to 10 ml with chloroform/methanol 9:1 (v/v) and two aliquots of 100 μl used for phospholipid quantitation. Phospholipid phosphorus was determined by the method of Bartlett\textsuperscript{17} after digesting the organic compounds at 190°C for 35 minutes in the presence of 500 μl 70% perchloric acid (w/v) and 200 μl 30% hydrogen peroxide (w/v). The amount of phospholipid in total samples was calculated from phospholipid phosphorus in the measured sample aliquots and the original sample volume (BAL fluid, P60000, S60000) or extract volume (total lung extract).

**ANALYSIS OF PHOSPHOLIPID CLASSES**

The distribution of total phospholipid classes in lipid extracts was determined by normal phase HPLC as described previously.\textsuperscript{15} In brief, aliquots containing 80–100 nmol phospholipid dissolved in chloroform/methanol (1:4 v/v) were resolved isocratically on an aminopropyl silica column (Nucleosil N H₂, 5 mm, Schambek, Germany) of 235 × 4.6 mm internal diameter. The mobile phase was delivered at a flow rate of 1 ml/min and comprised acetonitrile/methanol/water/methylphosphonic acid.
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Temperature. After washing the plates with TBS-alveolar epithelial cells revealed no detectable
containing 1 mg/ml BSA was added and al-
animals examined. Comparison of the number
signs of inflammation as leucocytic in-
ence intervals (CI) in parentheses. Statistical
40 mM choline chloride at a flow rate of 1 ml/
Hengoed, Mid Glamorgan, UK). PC molecular resuspended in 60
and second antibodies in a capture ELISA. across the bubble was measured by a pressure
-20, 0.05% (w/v) NaNO2, pH 7.4) containing 1 mg/ml bovine serum albumin for
were coated with chicken anti-human SP-A
medicals Ltd, High Wycombe, Bucks, UK) using the LaPlace equation.24
and New Zealand rabbits by repeated injection
were incubated for three hours at 37
standards (100
er, the samples and
the morphology of
and 4°C for lipid analysis. The 60 000g pellet (P60000) was
was used as a substrate for the phosphatase
was used as a substrate for the phosphatase
was used as a substrate for the phosphatase
respectively
acids base for
of washes with bu-
conjugate (Sigma Chemical Co. Ltd, Poole, Dorset, UK) diluted 1:10000 in TBS-NTC
conjugate (Sigma Chemical Co. Ltd, Poole, Dorset, UK) diluted 1:10000 in TBS-NTC
conjugate (Sigma Chemical Co. Ltd, Poole, Dorset, UK) diluted 1:10000 in TBS-NTC

ANALYSIS OF PHOSPHATIDYLCHOLINE
MOLECULAR SPECIES
The composition of individual molecular species
phosphatidylcholine (PC) was determined
A PC fraction was isolated from the total lipid extract on a 100 mg Varian Bondelut NH4
able cartridge (Jones Chromatography, Hengoed, Mid Glamorgan, UK). PC molecular species were then resolved on a Spherisorb
ODS II column (Shimadzu SFD, Bad Honnef, Germany) of internal diameter
250 x 4.6 mm at 50°C using a mobile phase of
methanol/water (92.5:7.5 v/v) containing 40 mM choline chloride at a flow rate of 1 ml/
in the BAL fluid were calculated from the
emission wavelength 440 nm) after post-col-

DETERMINATION OF TOTAL PROTEIN AND SP-A
Total protein was determined according to the method of Lowry et al.25 SP-A was determined by an ELISA technique using polyclonal anti-
form-free BAL fluid at 37°C or 4°C for functional analysis (see below).

PREPARATION OF SURFACTANT FROM BAL FLUID
Surfactant was prepared from cell-free BAL fluid by centrifugation at 60 000g and 4°C for one hour.22 The 60 000g supernatant (S60000) was harvested and frozen at –80°C for further lipid analysis. The 60 000g pellet (P60000) was resuspended in 60 µl 154 mM saline sup-
plemented with 1.5 mM calcium chloride and the phospholipid concentration determined from an aliquot and then adjusted to the desired concentration for functional analysis (see below).

FUNCTIONAL SURFACTANT ANALYSIS
For functional analysis P60000 was adjusted to 1.33 µmol phospholipid/ml with 154 mM saline/1.5 mM calcium chloride. Equilibrium
(γequil) and minimal (γmin) surface tensions were then determined using a pulsating bubble surfactometer (Electronetics Co, Amherst, New York, USA).23 Briefly, a bubble was cre-
ated in a surfactant suspension at 37°C and γequil was determined as surface tension 10
seconds after formation of the bubble. The bubble was then pulsed for five minutes at a frequency of 20 oscillations per min between a minimal bubble radius of 0.4 mm and a maximal radius of 0.55 mm. The pressure across the bubble was measured by a pressure
transducer. γequil and γmin were calculated using the LaPlace equation.24

DATA ANALYSIS
Data were expressed as means with 95% confi-
dence intervals (CI) in parentheses. Statistical
analyses were performed by one factor analysis
of variance (ANOVA) and the two tailed Stu-
dent’s t test using commercial software (Graph-
Pad InStat Version 1.1, San Diego, USA). Statistical values were corrected for multi-
group comparisons using the Bonferroni
method and statistical values of regression co-
efficients were calculated using the same soft-
w

RESULTS
HISTOLOGICAL EXAMINATION
The morphology of SP-A low expression (SP-A low) mice showed a regular structure of lung tissue (fig 1). No signs of inflammation as leucocytic infiltration or interstitial oedema were found in any of the animals examined. Comparison of the number
and distribution of Clara cells and type II
alveolar epithelial cells revealed no detectable
differences between MF1 and SP-A low expression (SP-A low) mice.
AMOUNT OF PHOSPHOLIPIDS AND SP-A

In BAL fluid from $cfr^{m1HGU/m1HGU}$ mice there were significant positive correlations between the amounts of phospholipid in BAL fluid or lavaged lung tissue and the body weight of the animals (fig 2). We therefore corrected the amount of phospholipid in BAL fluid for the individual mouse strains. After such correction the amount of phospholipid in total BAL fluid from $cfr^{m1HGU/m1HGU}$ mice was 43.4 (39.0 to 47.9) nmol/total BAL fluid/g body weight, while it was constantly lower in BAL fluid from control mice and $cfr^{m1HGU/m1HGU}$ mice (0.69 (0.63 to 0.75) μmol/total BAL fluid). Assuming that different body weights of the individual mouse strains contributed to the amount of phospholipid in the BAL fluid, we calculated the correlation between body weight and the amount of phospholipid in BAL fluid and lavaged lung tissue.
Table 1 Amounts of phospholipid (PL) and apparent surfactant protein A (SP-A) in BAL fluid and of phospholipid in lavaged lung tissue of cftr<sup>m1HGU/m1HGU</sup> mice versus individual healthy control strains

<table>
<thead>
<tr>
<th>PL (μmol/BAL fluid)</th>
<th>BALB/c (n = 11)</th>
<th>C57BL/6 (n = 13)</th>
<th>C3H/He (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.02 (0.99 to 1.16)</td>
<td>0.98 (0.86 to 1.2)</td>
<td>0.86 (0.71 to 1.05)</td>
<td>0.93 (0.82 to 1.06)</td>
</tr>
<tr>
<td>4.03 (3.86 to 4.22)</td>
<td>4.09 (3.90 to 4.28)</td>
<td>4.05 (3.85 to 4.24)</td>
<td>4.07 (3.88 to 4.25)</td>
</tr>
<tr>
<td>0.69 (0.63 to 0.75)</td>
<td>0.66 (0.60 to 0.72)</td>
<td>0.64 (0.59 to 0.69)</td>
<td>0.63 (0.58 to 0.68)</td>
</tr>
</tbody>
</table>

Data are means and 95% confidence intervals (in brackets) of the indicated numbers (n) of experiments.

* p<0.05, ** p<0.01, *** p<0.001 versus cftr<sup>m1HGU/m1HGU</sup> mice.

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Table 2 Phospholipid composition of bronchoalveolar lavage (BAL) fluid and lung tissue in cftr<sup>m1HGU/m1HGU</sup> mice versus MF1 and other control (BALB/c, C57BL/6, C3H/He) mice

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>cftr&lt;sup&gt;m1HGU/m1HGU&lt;/sup&gt; (n = 13)</th>
<th>MF1 (n = 25)</th>
<th>Other controls (n = 21)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>84.2 (81.2 to 87.1)</td>
<td>83.2 (81.2 to 86.2)</td>
<td>83.2 (81.2 to 86.2)</td>
</tr>
<tr>
<td>PG</td>
<td>11.1 (9.0 to 13.3)</td>
<td>12.0 (10.6 to 13.4)</td>
<td>10.4 (7.6 to 12.0)</td>
</tr>
<tr>
<td>SPH</td>
<td>1.1 (0.8 to 1.4)</td>
<td>0.8 (0.4 to 1.1)</td>
<td>1.1 (0.5 to 1.5)</td>
</tr>
<tr>
<td>PE</td>
<td>2.1 (1.2 to 2.9)</td>
<td>3.2 (2.1 to 4.5)</td>
<td>1.9 (1.0 to 2.7)</td>
</tr>
<tr>
<td>PS</td>
<td>1.6 (1.2 to 2.0)</td>
<td>0.7 (0.4 to 1.1)</td>
<td>2.1 (1.2 to 2.7)</td>
</tr>
<tr>
<td>Lung tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>51.3 (48.9 to 52.8)</td>
<td>50.3 (48.7 to 51.7)</td>
<td>49.4 (48.3 to 50.3)</td>
</tr>
<tr>
<td>PG</td>
<td>4.2 (3.2 to 5.4)</td>
<td>4.2 (3.2 to 5.4)</td>
<td>3.6 (3.1 to 4.2)</td>
</tr>
<tr>
<td>SPH</td>
<td>8.2 (7.9 to 8.5)</td>
<td>8.4 (8.0 to 8.7)</td>
<td>8.3 (8.0 to 8.6)</td>
</tr>
<tr>
<td>PE</td>
<td>31.4 (29.9 to 32.6)</td>
<td>36.2 (34.1 to 38.5)</td>
<td>36.7 (35.5 to 37.9)</td>
</tr>
<tr>
<td>PS</td>
<td>2.2 (1.8 to 2.6)</td>
<td>1.2 (1.0 to 1.4)</td>
<td>2.2 (1.8 to 2.7)</td>
</tr>
</tbody>
</table>

Data are means and 95% confidence intervals (in brackets) of the indicated numbers (n) of experiments.

Table 3 Molecular species of phosphatidylcholine (PC) in bronchoalveolar lavage (BAL) fluid and lung tissue in cftr<sup>m1HGU/m1HGU</sup> versus MF1 and other control (BALB/c, C57BL/6, C3H/He) mice

<table>
<thead>
<tr>
<th>PC species</th>
<th>cftr&lt;sup&gt;m1HGU/m1HGU&lt;/sup&gt; (n = 9)</th>
<th>MF1 (n = 14)</th>
<th>Other controls (n = 17)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC16:0/14:0</td>
<td>5.3 (4.9 to 5.8)</td>
<td>6.7 (5.3 to 8.1)</td>
<td>6.7 (5.3 to 8.1)</td>
</tr>
<tr>
<td>PC16:0/16:0</td>
<td>57.9 (56.4 to 59.4)</td>
<td>58.3 (57.3 to 59.9)</td>
<td>58.3 (56.0 to 60.7)</td>
</tr>
<tr>
<td>PC16:0/18:2</td>
<td>19.8 (18.8 to 20.9)</td>
<td>17.9 (16.9 to 18.9)</td>
<td>13.5 (10.3 to 16.8)*</td>
</tr>
<tr>
<td>PC16:0/18:4</td>
<td>4.5 (4.1 to 5.0)</td>
<td>5.2 (5.0 to 5.4)</td>
<td>7.2 (4.0 to 8.0)**</td>
</tr>
<tr>
<td>PC16:0/18:6</td>
<td>6.3 (5.6 to 6.9)</td>
<td>6.9 (6.5 to 7.2)</td>
<td>9.0 (8.0 to 9.6)**</td>
</tr>
<tr>
<td>PC18:0/18:0</td>
<td>0.4 (0.2 to 0.6)</td>
<td>0.2 (0.1 to 0.3)</td>
<td>1.2 (0.4 to 2.0)</td>
</tr>
<tr>
<td>PC18:0/18:2</td>
<td>1.1 (0.9 to 1.2)</td>
<td>1.2 (0.6 to 2.7)</td>
<td>1.4 (1.1 to 1.7)</td>
</tr>
<tr>
<td>PC18:0/18:4</td>
<td>0.4 (0.2 to 0.6)</td>
<td>0.2 (0.1 to 0.3)</td>
<td>0.8 (0.5 to 1.2)</td>
</tr>
<tr>
<td>Others</td>
<td>3.9 (2.9 to 4.9)</td>
<td>3.7 (3.3 to 4.1)</td>
<td>3.4 (2.7 to 4.3)</td>
</tr>
</tbody>
</table>

Data are means and 95% confidence intervals (in brackets) of the indicated numbers (n) of experiments.

Figure 2 Correlation between the amounts of lung phospholipid and body weight. Linear regression curves were calculated between the total amount of phospholipids in (A) BAL fluid or (B) lavaged lung tissue from cftr<sup>m1HGU/m1HGU</sup> mice (n = 25) and control mice (C57BL/6, n = 37) and the individual body weights of the mice. Thick bars represent regression curves while thin cftr<sup>m1HGU/m1HGU</sup> mice or dotted (control) lines represent 95% confidence intervals. In BAL fluid of cftr<sup>m1HGU/m1HGU</sup> mice r = 0.591, p < 0.01; controls r = 0.671, p < 0.0001. In lung tissue of cftr<sup>m1HGU/m1HGU</sup> mice r = 0.458, p < 0.05; controls r = 0.826, p < 0.00001.
Figure 3  Concentrations of phospholipids in P60000 (■) and S60000 (○) of BAL fluid of cftr<sup>m1HGU/m1HGU</sup> mice, MF1, and other control mice. BAL fluid was centrifuged for one hour at 60 000 g and at 4°C. Phospholipid concentrations were determined in the pellet (P60000) and supernatant (S60000) as described in the Methods section. cftr<sup>m1HGU/m1HGU</sup> (n = 6); MF1 (n = 3); other control mice: BALB/c (n = 4); C57BL/6 (n = 3); C3H/He (n = 5). Bar height represents 95% confidence interval. * p < 0.05 versus MF1 and other controls.

### Table 4  Surface tension function (in mN/m) of 60000g pellet (P60000) from bronchoalveolar lavage (BAL) fluid in cftr<sup>m1HGU/m1HGU</sup> versus MF1 and other control (BALB/c; C57BL/6; C3H/He) mice

<table>
<thead>
<tr>
<th>PC species</th>
<th>cftr&lt;sup&gt;m1HGU/m1HGU&lt;/sup&gt; (n = 7)</th>
<th>MF1 (n = 7)</th>
<th>Other controls (n = 13)&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>γequil</td>
<td>24.7 (19.1 to 30.3)</td>
<td>25.0 (24.2 to 25.9)</td>
<td>28.6 (25.5 to 31.6)</td>
</tr>
<tr>
<td>γmin</td>
<td>17.4 (14.4 to 20.5)</td>
<td>21.6 (20.0 to 23.1)</td>
<td>18.2 (13.4 to 22.9)</td>
</tr>
<tr>
<td>Pulsation 1</td>
<td>15.6 (11.3 to 19.8)</td>
<td>18.0 (13.1 to 22.9)</td>
<td>16.6 (10.8 to 22.4)</td>
</tr>
<tr>
<td>Pulsation 9</td>
<td>11.1 (5.3 to 16.8)</td>
<td>13.6 (7.0 to 20.2)</td>
<td>15.5 (10.1 to 20.9)</td>
</tr>
<tr>
<td>Pulsation 100</td>
<td>2.8 (0.1 to 5.5)</td>
<td>3.2 (0.1 to 6.7)</td>
<td>2.6 (1.7 to 3.5)</td>
</tr>
</tbody>
</table>

<sup>1</sup> 3 experiments from BALB/c (n = 4), C57BL/6 (n = 5), and C3H/He (n = 4) mice.

Table 4 shows the surface tension function (in mN/m) of 60000g pellet (P60000) from bronchoalveolar lavage (BAL) fluid in cftr<sup>m1HGU/m1HGU</sup> versus MF1 and other control (BALB/c; C57BL/6; C3H/He) mice. The relative concentration of active surfactant isolated by differential ultracentrifugation at 60 000g (P60000) compared with the phospholipids in the supernatant (S60000) of the BAL fluid is shown in fig 3. In BAL fluid from cftr<sup>m1HGU/m1HGU</sup> mice the amount of phospholipid in P60000 was significantly higher than in controls. To evaluate whether the surface tension function of the P60000 was different from that of controls we measured the surface tension function of this material. Equilibrium surface tension (γequil) after 10 seconds of static adsorption of P60000 from cftr<sup>m1HGU/m1HGU</sup> mice reached values below 5 mN/m as quickly as surfactant from the control mice (table 4).

### COMPOSITION OF PHOSPHOLIPID CLASSES AND PHOSPHATIDYLCHOLINE (PC) MOLECULAR SPECIES

Investigation of phospholipid compositional changes in BAL fluid and lung tissue of cftr<sup>m1HGU/m1HGU</sup> mice was in the first instance addressed by HPLC analysis of phospholipid classes. As shown in table 2 there were no differences in the relative composition of phospholipid classes in BAL fluid and lung tissue of cftr<sup>m1HGU/m1HGU</sup> and control mice. Importantly, the contributions of PC and phosphatidyglycerol (PG) were not decreased, nor were those of sphingomyelin (SPH) or phosphatidylethanolamine (PE) increased in either BAL fluid or lung tissue of cftr<sup>m1HGU/m1HGU</sup> mice. Because there were no differences in the phospholipid compositions of BALB/c, C57BL/6 and C3H/He mice, these data were combined and are shown as “other controls”. Since the key molecule of surface tension function of lung surfactant is PC16:0/16:0, and since alterations in the concentrations of individual molecular species of PC are a more sensitive indicator of pulmonary surfactant alterations than the determination of PC fatty acids by gas chromatography, we subsequently investigated these PC molecular species in both BAL fluid and lavaged lung tissue. The BAL fluid and lavaged lung tissue of cftr<sup>m1HGU/m1HGU</sup> mice predominantly contained PC16:0/16:0 (table 3), comprising 57.9 (56.4 to 59.4) mol% and 34.8 (33.2 to 36.3) mol% of total PC, respectively. These values were identical to those from control mice. Other typical components of surfactant – for example, palmitolymyristoyl-PC (PC16:0/14:0) and palmitoyl-palmitoleoyl-PC (PC16:0/16:1) – were also without major changes in cftr<sup>m1HGU/m1HGU</sup> mice compared with controls. The highly unsaturated PC species palmitoylethanolaminouyl-PC (PC16:0/20:4), stearolylarachidonoyl-PC (PC18:0/20:4), and palmitoyldocosahexaenoyl-PC (PC16:0/22:6) were not detected in significant amounts in BAL fluid but made up 12–14% in lung tissue. Again, there were no major differences between cftr<sup>m1HGU/m1HGU</sup> and control mice.

### Discussion

Cystic fibrosis is an inherited disease with a defect in the CFTR gene resulting in the expression of a defective CAMP-dependent chloride channel. Previous reports of impairment to the biophysical properties of airway secretions, as well as lipid extracts of such secretions in patients with cystic fibrosis as-
surfactant (large aggregates) rather than an accumulation together with a decreased SP-A to phospholipid ratio in patients with cystic fibrosis. Hence, lung surfactant metabolism could be affected by the basic defect of cystic fibrosis prior to and independent from the chronic inflammatory processes in the lungs. To address this issue we employed the \( \text{cftr}^{+/+} \) mouse as an animal model to study aspects of lung surfactant metabolism in cystic fibrosis prior to chronic pulmonary infection. The \( \text{cftr}^{+/-} \) mouse is a useful model for human cystic fibrosis because, unlike the absolute null mutant mouse, this model shows normal growth rate and good long term survival. This is due to the “leaky” nature of the mutation which results in 5–10% residual wild type after expression. The \( \text{cftr}^{-/-} \) mouse is thus analogous to patients with cystic fibrosis carrying CFTR mutations which retain partial CFTR function.

Our data show, for the first time, that BAL fluid concentrations of surfactant phospholipid as an index for the intra-alveolar surfactant pool are primarily increased by about 60% in a CFTR-deficient organism compared with all other tested control strains, whereas the amount of phospholipid in lung tissue is not altered. While we could not demonstrate biochemical alterations of alveolar phospholipid material, the quantitative changes in alveolar surfactant phospholipids were due to an increase in material which could be pelleted by ultracentrifugation at 60 000g (P60000) as previously described in the mouse by other investigators. In that study it was demonstrated that the P60000 consisted of large surfactant aggregates, while so-called small surfactant aggregates remained in the 60000g supernatant (S60000). Moreover, it was demonstrated that P60000 consisted of surface active material while S60000 contained only inactive material. Our functional data are consistent with these observations since P60000 present in BAL fluid from both \( \text{cftr}^{+/+} \) and \( \text{cftr}^{-/-} \) mice was depopulated by about 60% in described, we did not detect such changes in BAL fluid from \( \text{cftr}^{-/-} \) mice without infection. Similarly, no impairment of surface tension function of the surfactant isolated from the BAL fluid of \( \text{cftr}^{-/-} \) mice was detected. We therefore conclude that such alterations are due to chronic inflammation and leucocyte infiltration of cystic fibrosis lungs, secondary to colonisation with opportunistic bacteria. Our data support the concept that any alteration in the phospholipid composition is due to chronic infection and inflammation rather than an effect of CFTR mutations.

While phospholipids were increased in the BAL fluid, the amount of SP-A remained unchanged. This unexpected finding is in agreement with a recent study by Doyle et al who showed that phospholipid and SP-A secretion into the alveolus are independently regulated. While the mechanisms of such changes in \( \text{cftr}^{-/-} \) mice are still uncertain and await further investigation, the potential consequences for pulmonary defence of an increased amount of phospholipid and a decreased ratio of SP-A to phospholipid are more obvious. Surfactant contributes to pulmonary integrity as a first line of defense. Phospholipid inhibits the activity of SP-A to opsonise bacteria and depresses the respiratory burst of alveolar macrophages upon challenge. Increased phospholipid concentrations together with a decreased SP-A to phospholipid ratio in \( \text{cftr}^{-/-} \) mice, and possibly in patients with cystic fibrosis, may
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impair the resistance of the lungs against bacteria. Since human SP-A was used as a standard in the immunoassay, the values obtained for the mouse SP-A are not absolute and are only for use for the comparison of SP-A levels within the different strains of mice examined in this study. Nevertheless, our results are consistent with recent findings on BAL fluid from patients with cystic fibrosis which showed an increased concentration of PC, while the ratio between SP-A and phospholipid was even more decreased in the BAL fluid of patients with cystic fibrosis than in that of cftrHGUmHG mice. However, while the data from human BAL fluid originate from lungs that already show symptoms of chronic impairment and, possibly, proteolytic degradation of SP-A due to inflammation, the cftrHGUmHG mice used in this study were kept under specific pathogen-free conditions. Histological examination of lungs confirmed the absence of leukocytic infiltrations or other visible alteration of lung structure and cellular distribution, and the absence of increases in membrane phospholipids such as PE or SPI in BAL fluid from cftrHGUmHG mice provides further evidence of minimal damage to their lungs. It is therefore possible that the greater decrease in the SP-A to phospholipid ratio in BAL fluid from patients with cystic fibrosis compared with cftrHGUmHG mice may in part be due to secondary degradation of SP-A caused by chronic inflammation of the lung.

We conclude that, in cftrHGUmHG mice without chronic inflammatory lung disease, the composition of phospholipid classes and phosphatidylinositol molecular species, the concentration of SP-A, and the surface tension function of surfactant are all unchanged compared with control mice. However, the total amount of phospholipid and the ratio of phospholipid to SP-A was increased in the BAL fluid, possibly as a result of altered surfactant function of innate lung defense against pathogens. Biol Neonate 1995; 67(Suppl 1):2-17.


Lung surfactant in a cystic fibrosis animal model: increased alveolar phospholipid pool size without altered composition and surface tension function in cftrm1HGU/m1HGU mice.
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