Impaired recycling of surfactant-like liposomes in type II pneumocytes from injured lungs

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Background: Surfactant synthesis and secretion has been shown to be impaired in type II cells from diseased lungs. The mechanism of surfactant lipid recycling, which is an important physiological process in surfactant treatment, was studied in type II cells isolated from injured lungs.

Methods: Different stages of lung injury were induced by exposing rats to 10 ppm nitrogen dioxide (NO₂) for 3, 20, and 28 days. Type II cells were isolated from these lungs and recycling of ³H-DPPC labelled surfactant-like liposomes was studied in vitro.

Results: Uptake of liposomes (150 µg/ml) for 20 minutes in the absence and presence of surfactant protein-A (SP-A, 5 µg/ml) was higher in cells from NO₂ injured lungs (63–78%) than in control cells. There was no difference in liposome uptake between the groups with NO₂ exposure of different duration. After liposome uptake, most of the internalised label remained in the phosphatidylcholine (PC) fraction and increased with duration of exposure to NO₂. After 20 minutes internalisation, cells were allowed to resecrete lipids for a further 20 minute period. In control cells and from all stages of lung injury, liposomes that had been internalised in the presence of SP-A were resecreted to a greater extent than those internalised without SP-A. However, cells from lungs exposed to NO₂ resecreted less lipid than cells from control lungs. Again, there was no difference in resecretion between the groups with NO₂ exposure of different duration.

Conclusion: Type II cells from injured lungs internalise more surfactant-like liposomes than cells from controls, suggesting a putative therapeutic significance to cope with limited alveolar surfactant pools in lung injury.

Methods

Animals, NO₂ exposure and experimental groups

All experiments were carried out on specific pathogen free adult male Sprague Dawley rats (body weight 170–180 g) obtained from Charles River Wiga (Sulzfeld, Germany). Every experiment was done with 8–12 animals. Each experimental group consisted of four animals that were kept in one exposure chamber with free access to food and water.

Nitrogen dioxide atmospheres were generated by mixing compressed air with NO₂ from a tank (Messer-Griesheim, Duisburg, Germany). The final concentration for all exposure experiments was 10 ppm. Normal air breathing animals were used as controls because we have found earlier that there is no difference in these and those exposed to compressed air.
Chemicals
All reagents were purchased from Sigma (Deisenhofen, Germany) unless stated otherwise. The Dulbecco's modified Eagles's medium (DMEM) for culture of type II cells was supplied by Life Technology (Karlruhe, Germany) and radiolabelled lipids were purchased from Amersham (Braunschweig, Germany). For resecretion experiments liposomes (mannum Schwäbisch-Gemünd, Germany) at 40% maximal out-mixture was pulse sonicated (Brenson B12 Sonofier, Heineheim Schwäbisch-Gemünd, Germany) at 4°C for 10 minutes and the resulting fractions were kept for further determinations. For studies of surfactant-like liposome uptake, unilamellar liposomes were prepared as described above with the following composition: 5.4 mg DPPC, 1 mg PG, 2.5 mg egg yolk PC, 1 mg cholesterol, and 0.1 mg cholesterole oleate in chloroform. This mixture was supplemented with H-DPPC (specific activity 87 µCi/mg) to a final radioactivity of 1.46 nCi/µg lipid and with 14C-cholesterol oleate (specific activity 115 nCi/mg) to a final radioactivity of 1 nCi/µg lipid. The mixture was stored at 4°C and used within 1 week. Before use the liposomes were centrifuged at 1000g to remove larger aggregates.

Isolation of type II pneumocytes
Rats were anaesthetised with pentobarbital sodium (50 mg/kg) mixed with 100 IU heparin sodium and injected intraperitoneally. When the animals were in deep anaesthesia the trachea was cannulated and, after thoracotomy, the vena cava was cut and the lungs perfused free of blood via the pulmonary artery and removed from the body. The lungs were washed extracorporally with five volumes of 8 ml normal saline (37°C) saline; after instillation of each volume the fluid was allowed to run out passively. To obtain alveolar cells and debris free lavage the fluid was centrifuged at 300g and 4°C for 10 minutes and the resulting fractions were kept for further determinations.

Isolation of type II cells was done following the procedure described by Dobbs and coworkers. In brief, after bronchoalveolar lavage the lungs were washed with the solutions described by Dobbs before elastase solution was instilled. Digestion with elastase was allowed to take place at 37°C for 20 minutes before the large airways were removed; in the presence of DNase I (250 µg/ml) the lungs were minced with scissors and the elastase reaction was then stopped by addition of 5 ml fetal bovine serum per lung (Gibco-BRL, Eggenstein, Germany). The final cell suspension was filtered several times through gauze nylon and washed by gentle centrifugation. The cell pellet was resuspended in DMEM and transferred to rat immunoglobulin G coated bacteriological Petri dishes to a density of 30×106 cells. After incubation for 1 hour at 37°C in a 10% CO2-air incubator, the macrophages were removed, centrifuged, and used for further experiments.

Purity and viability
After cell isolation, type II cell viability was determined by trypan blue dye exclusion. The purity of the cell preparation was evaluated with the Papanicolaou stain. Samples of every exposure condition were evaluated per microscope field in triplicate and an average value was determined.

Isolation of lamellar bodies
The isolation of lamellar bodies was performed as outlined elsewhere. In brief, type II cells were homogenised in 0.32 M sucrose in 10 mM Tris/NaCl buffer and layered over a discontinuous gradient of 0.45 M and 0.55 M sucrose in Tris/NaCl buffer. After centrifugation at 90 000g for 3 hours, the band at the interface between 0.45 M and 0.55 M sucrose was collected. The lamellar body was obtained after dilution of the interface to 0.2 M sucrose followed by centrifugation at 20 000g for 15 minutes.

Liposome preparation
For studies of surfactant-like liposome uptake, unilamellar liposomes were prepared as described by Wissel et al, mixing 5.5 mg 1,2-dipalmitoyl-1-3-phosphatidyl-N-(methyl-H) choline (DPPC), 1 mg PG, 2.5 mg egg yolk PC, and 1 mg cholesteryl in chloroform. This mixture was supplemented with H-DPPC (specific activity 87 µCi/mg) to a final radioactivity of 1.46 nCi/µg lipid. After evaporation of the chloroform, 1 ml of the described mixture was pulse sonicated (Brenson B12 Sonofier, Heinemann Schwäbisch-Gemünd, Germany) at 40% maximal output for 20 minutes. For resecretion experiments liposomes were prepared as described above with the following composition: 5.5 mg DPPC, 1 mg PG, 2.5 mg egg yolk PC, 1 mg cholesteryl, and 0.1 mg cholesteryl oleate in chloroform. This mixture was supplemented with H-DPPC (specific activity 87 µCi/mg) to a final radioactivity of 1.46 nCi/µg lipid and with 14C-cholesterol oleate (specific activity 115 nCi/mg) to a final radioactivity of 1 nCi/µg lipid. The mixture was stored at 4°C and used within 1 week. Before use the liposomes were centrifuged at 1000g to remove larger aggregates.

Liposome uptake into isolated type II cells
Liposome uptake was studied using freshly isolated type II cells in floating culture. Cell culture tubes with 2.5×106 cells in 1 ml DMEM containing 0.1% (w/v) lipid free bovine serum albumin (BSA) were incubated with 9 µl of the described liposome suspension (see above) in the absence or presence surfactant protein-A (SP-A, 5 µg/ml) over 60 minutes at 37°C. Samples were taken every 10 minutes to determine the kinetics of liposome uptake. Cells were sedimented by centrifugation (160g, 4°C, 10 minutes) and resuspended in DMEM containing 5% fat free BSA and 10 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N''-tetraacetic acid (EGTA). Sedimentation and resuspension were repeated twice in DMEM containing 0.1% BSA and 10 mM EGTA and a final washing procedure with only DMEM. Lipid material still associated with the type II cells after this rigorous washing procedure is termed “internalised lipids” or lipid uptake.

Thin layer chromatography
To facilitate recovery and determination of internalised liposomes, carrier lipids (organic lipid extract from pig lung homogenate, 600 µg, dissolved in 100 µl chloroform/methanol 2:1) were added to the samples. After lipid extraction, phospholipids were separated by two dimensional thin layer chromatography (TLC) on silica H60 TLC plates (Merck, Mannheim, Germany) as described by Wissel et al. The phospholipid spots were visualised by brief exposure to iodine vapour, scraped into 6 ml Optifluor (Packard, Groningen, Netherlands) scintillation fluid, and counted for radioactivity. For experiments comparing liposome uptake into cells from lungs exposed to NO2 for different durations, an uptake period of 30 minutes was used. Because the amount of phospholipid from the single experiments was low, phospholipids were only analysed for the PC and “other lipids” moieties.

Surfactant resecretion from isolated type II pneumocytes
After liposome uptake, the radioactivity in the washed type II cells represents the net liposome uptake—that is, 100% radioactivity at time point zero for the resecretion process.

As in the studies of liposome uptake, an optimal period of resecretion had to be determined with kinetic studies. After the liposome internalisation process, cells from control animals and from those exposed to NO2 were resuspended in 1 ml DMEM containing 0.1% BSA and incubated at 37°C for 60 minutes. Samples were taken every 10 minutes to determine the resecreted lipid in the media and the remaining label in the cells. The resecretion process was terminated by addition of 1 ml ice cold DMEM. Further cell washing proceeded as described previously. All supernatants were pooled and kept for determination of the resecreted radioactive material. Phospholipid extraction was performed as described for liposome uptake.

Isolation of surfactant associated protein (SP-A)
Surfactant associated protein A (SP-A) was isolated from lung lavages from normal control rats using the method of Hawgood and coworkers. Protein purity was analysed in SDS-PAA gels and bioactivity as the capacity to inhibit phosphatidylycholine secretion from isolated type II cells.

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Other methods
Protein was measured with the Bio-Rad reagent (Munich, Germany) and phospholipid was calculated according to standard procedures. Lactate dehydrogenase (LDH) activity was measured in cells and culture media by conversion of β-nicotinamide adenine dinucleotide (β-NAD).

Statistical analysis
Assays were performed in triplicate for each sample from each exposure condition and a mean value was determined. The mean values for each sample were then used for analysis. The results are expressed as mean (SE) values. For paired analyses between two groups the Student’s t test was used. Statistical significance between more than two groups was tested using the ANOVA procedure and subsequent Scheffé test. p values of ≤0.05 were considered significant.

RESULTS
Type II cell preparations, cell yield, purity and viability
Evaluation of in vitro surfactant-like liposome uptake and lipid resecretion from type II pneumocytes from lungs exposed to NO₂ for different durations was only performed on freshly isolated cells. As previously reported, the yield of type II pneumocytes from NO₂ exposed lungs was significantly higher than that from control lungs (data not shown). Cell purity determined by Papanicolaou stain was comparable for all cell preparations and ranged from 91% to 93% (n=44 cell isolations). Cell viability as evaluated by trypan blue dye exclusion was also comparable in all preparations (range 95–96%, n=44 isolations).

Kinetics of liposome association
To determine an incubation period for a comparison of the association of surfactant-like liposomes with type II cells from all experimental groups (0, 3, 20, and 28 days of NO₂ exposure), the uptake kinetics were evaluated at 37°C over a 60 minute time period. During this period type II pneumocytes (2.5 × 10⁶ cells/ml) were incubated in DMEM with ¹H-DPPC containing liposomes (150 µg/ml) in the absence and presence of 5 µg/ml SP-A. Cell aliquots were taken from the samples every 10 minutes, subjected to the rigorous washing procedure to remove lipid adherent to the cells, and assayed for associated radioactivity.

![Figure 1](https://example.com/f1.png)

**Figure 1** Time course of uptake of ¹H-DPPC labelled liposomes by freshly isolated type II cells from control animals and those exposed to 10 ppm NO₂ for 3 days. Type II cells were isolated from lungs of controls and from lungs with different stages of lung injury induced by exposure to atmosphere containing 10 ppm NO₂ for 3, 20, and 28 days. Because the graphs of uptake kinetics for the cells exposed to NO₂ for 20 and 28 days overlapped with those of the 3 day exposure, they were not included in the figure. Values are mean (SE), n=5; *p<0.05, **p<0.01 difference between time points of the 3 day exposure to NO₂ and corresponding time points of control lungs in the absence and presence of SP-A.

In cells isolated from the lungs of control animals in the absence of SP-A, the amount of ¹H-labelled liposomes increased faster at early time points (10, 20, and 30 minutes) than at 40 and 60 minutes (fig 1). Liposome association with cells from lungs exposed to NO₂ for 3 days followed the same kinetics, but the amount of cell associated lipid for all time points measured was significantly increased compared with control cells. Liposome uptake into cells exposed to NO₂ for 20 and 28 days had the same kinetics as those for cells exposed for 3 days (because the graphs overlapped with the 3 day NO₂ exposure data curve they were not included in the figure). When liposome uptake was performed in the presence of SP-A (5 µg/ml), the cell associated label followed the same kinetics but was about three times higher than in the experiments without SP-A. At 20 minutes the cell associated liposome label was about half maximal and this was therefore regarded suitable for further comparative studies.

Is liposome association “adherence to cell membranes” or “cellular uptake”?
To determine whether labelled liposomes were internalised into cells or just adhered to the cell membranes, the incubation assay was also performed at low temperature (4°C) to block energy dependent active uptake processes. After incubation for 20 minutes a considerable amount of strongly adherent liposome material was attached to the cells that could not be removed by normal DMEM washings. Using the rigorous washing procedure (three washing cycles in DMEM supplemented with 5% fat free BSA and 10 mM EGTA solution followed by two washing cycles in DMEM with 0.1% BSA), the adherent liposome material was reduced to 40% of the attached material. Further washings did not remove any additional liposomes. The labelled liposomes that were resistant to the rigorous BSA-EGTA washing procedure are therefore considered as cellular liposome uptake in this study.

After the washing procedure a considerable amount of label from the liposomes was associated with the cells at 4°C, suggesting that lipid exchange makes a significant contribution to the apparent lipid uptake or non-calcium dependent adherence. Because of this possibility, liposomes were labelled with cholesterol oleate, a lipid that does not easily exchange and suggested elsewhere. For all experimental groups liposome uptake in the absence of SP-A did not differ whether it was performed at 4°C or 37°C (table 1). When SP-A was included in the uptake experiments at 4°C, comparable values were obtained to those in the experiments without SP-A at 4°C and 37°C. A clear increase in liposome uptake was observed when SP-A mediated uptake was performed at 37°C, resulting in an increase of label in the cells from control and from NO₂ exposed lungs of 2–3 fold.

Lipid uptake into cells from diseased lungs
As shown in fig 2, after incubation for 20 minutes at 37°C, uptake of ¹H-DPPC labelled liposomes into type II cells from the different periods of NO₂ exposure (3, 20, and 28 days) was increased compared with uptake by control cells (p<0.05 for 3, 20 and 28 days vs no exposure). The increase was similar for all periods of NO₂ exposure and was 68–81% higher than that of control cells; there was no difference between the different NO₂ exposure groups. When SP-A (5 µg/2.5 × 10⁶ cells) was included in the incubation medium, uptake of DPPC labelled liposomes into cells from control and NO₂ exposed lungs was more than twice that without SP-A. Internalisation into cells from NO₂ exposed lungs was 63–78% more than that of control cells, with no difference between the different NO₂ exposure groups.

As already described, at low temperatures an increased ¹H-DPPC liposome label was associated with the cells, suggesting the possibility of lipid exchange to the apparent uptake. To determine whether the observed lipid uptake was...
due, at least in part, to lipid exchange, uptake experiments were performed with \(^{14}\)C-cholesterol ester labelled liposomes, a lipid marker that does not exchange easily.

As shown in fig 3, the results from these experiments were comparable to those with \(^{3}\)H-DPPC labelled liposomes. For all NO\(_2\) exposed groups in the absence of SP-A, a 75% increase in \(^{14}\)C-cholesterol ester labelled liposome uptake was found compared with cells from control lungs. In the presence of SP-A, the uptake of \(^{14}\)H-cholesterol ester labelled liposomes was about 250% higher than in the absence of SP-A. Cells from NO\(_2\) exposed lungs incorporated about 75% more cholesterol ester than cells from the unexposed lungs.

To confirm cell viability after liposome incubation, cells were assayed by trypsin blue dye exclusion and by measurement of lactate dehydrogenase (LDH) activity in the incubation media. Cell viability was comparable in all experimental groups with a range of 93–97% (n=38 experiments). In all incubation experiments after 20 minutes of liposome uptake, LDH activity in the media was less than 2% of the total cellular activity (n=38 experiments).

**Liposome label in lamellar bodies after NO\(_2\) exposure**

After liposome uptake into type II cells, the cell associated label was higher in cells that had been incubated with liposomes in the presence of SP-A than in those incubated in the absence of SP-A. To determine the extent to which radioactivity was contained in the lamellar bodies, these subcellular elements were isolated from the 0.45 M sucrose gradient centrifugation fraction and determined for radioactivity.

It was found that liposome uptake resulted in higher lipid label in the lamellar bodies in the presence of SP-A than in the absence of SP-A (table 2). This SP-A mediated increased uptake was threefold for type II cells from controls and twofold for cells from the different NO\(_2\) exposure groups. Even in the absence of SP-A, cells from NO\(_2\) exposed animals incorporated more label into the lamellar bodies than cells from control animals. There was no difference in the radioactivity of the lamellar bodies in cells from control animals and those in the different NO\(_2\) exposure groups.

**Kinetics of lipid resecretion**

To ascertain whether type II cells from the different NO\(_2\) exposure groups resecrete lipids from the internalised liposomes, an optimal resecretion period had to be determined to allow comparison between all experimental groups. Freshly isolated type II pneumocytes were incubated in the presence and absence of SP-A (5\(\mu\)g/ml) for 20 minutes at 37\(^\circ\)C with \(^{14}\)H-DPPC labelled liposomes as described earlier. For all experimental groups the amount of internalised \(^{14}\)H-DPPC labelled liposomes was set at 100%. After liposome uptake, cells were washed with BSA-EGTA supplemented DMEM to remove adherent lipid and incubated in lipid free medium for 60 minutes. During this period type II cells were allowed to resecrete the incorporated lipid material. Resecretion was calculated as the percentage of rescreted label in the medium and the label in the medium plus that in the cells.

Resecretion kinetics showed that, in cells from control lungs and those exposed to NO\(_2\) for 3 days, lipids internalised in the absence of SP-A were resecreted over 60 minutes only to a small extent (10–15% of internalised material; fig 4). Cells from lungs exposed to NO\(_2\) for 20 and 28 days followed the same kinetics (graphs were not included in the figure because of overlapping with the 3 day NO\(_2\) exposure curve). There was no obvious difference in lipid resecretion for cells from control lungs and those from NO\(_2\) exposed lungs. In contrast, lipids

### Table 1: Temperature dependency of liposome uptake by type II pneumocytes isolated from lungs exposed to 10 ppm NO\(_2\) under different conditions

<table>
<thead>
<tr>
<th></th>
<th>-SP-A</th>
<th>+SP-A</th>
<th>-SP-A</th>
<th>+SP-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>37°C</td>
<td>4°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Control</td>
<td>146.3 (9.8)</td>
<td>168.5 (13.9)</td>
<td>192.1 (3.3)</td>
<td>477.8 (12.9)**</td>
</tr>
<tr>
<td>3 days</td>
<td>190.0 (6.5)</td>
<td>240.0 (11.0)</td>
<td>202.9 (5.6)</td>
<td>542.4 (5.7)**</td>
</tr>
<tr>
<td>20 days</td>
<td>180.0 (7.7)</td>
<td>204.4 (9.9)</td>
<td>176.9 (10.2)</td>
<td>547.7 (12.6)**</td>
</tr>
<tr>
<td>28 days</td>
<td>246.0 (11.4)</td>
<td>314.4 (13.6)</td>
<td>263.8 (7.9)</td>
<td>800.3 (10.7)**</td>
</tr>
</tbody>
</table>

Values are mean (SE) pmol cholesterol oleate/mg protein; n=3–4 experiments.

*\(p<0.01\) difference between time points of the 3 day exposure to NO\(_2\) and corresponding time points of controls in the absence and presence of SP-A.
that had been internalised in the presence of SP-A were resecreted to a greater extent. The intracellular amount decreased significantly with time until 20 minutes resecretion. At this time point resecretion from cells of control lungs had reached a maximum (59% of the intracellular label). After that period the intracellular label increased again, reaching 74% of the original amount of internalised label. The cells from the NO2 exposed lungs followed the same kinetics (graphs for 20 and 28 day NO2 exposure not included in the figure because of overlapping). However, type II cells from NO2 exposed lungs resecreted less lipid material than cells from control lungs. Because maximum resecretion occurred in all experimental groups at 20 minutes, this time point was regarded as suitable for comparison of lipid resecretion between all experimental groups.

**Lipid resecretion from type II cells of diseased lungs**

As shown in fig 5, at 37°C resecretion of DPPC that was internalised in the absence of SP-A was significantly reduced from lungs exposed to NO2 for 3, 20, and 28 days compared with controls (p<0.01 for 3, 20, and 28 days vs controls). No differences were observed between the different NO2 exposure groups. Up to 58% of the amount of liposomes that were internalised by control cells were resecreted compared with 10% in cells from the NO2 exposed lungs. From the ‘H-DPPC labelled lipids that were internalised in the presence of SP-A, a larger quantity was resecreted from the lipids taken up without the protein. Furthermore, for resecretion of the SP-A mediated internalised liposomes it was found that lipid resecretion was lower in cells from NO2 exposed lungs than from controls, but the rate of resecretion did not differ between exposure groups.

Cell viability as evaluated by trypan blue dye exclusion exhibited a viability of 95–98% for all experimental groups. In addition, LDH activity in the cell culture media of the resecretion experiments was less than 3% of the total cellular activity.

**Distribution of label from ‘H-DPPC incorporated liposomes among lipid classes and fate of resecreted lipids**

To determine whether ‘H-DPPC labelled liposomes were metabolised by type II cells during the uptake period, the distribution of the intracellular label was determined. After phospholipid extraction and two dimensional TLC, phosphorus was measured and radioactivity associated with the PC moiety and other phospholipid moieties determined. For all NO2 exposed groups, incorporation of ‘H-DPPC into the PC moiety was significantly increased compared with uptake into the PC fraction of control cells (table 3).

To determine whether lipids from ‘H-DPPC labelled liposomes that were internalised in the absence and presence of SP-A were modified by type II cells from various stages of NO2 induced lung injury, the distribution of the label in the PC moiety and in other phospholipid moieties was determined. Freshly isolated type II cells were allowed to internalise and resecrete lipids as described above. After resecretion, phospholipids were extracted from the cells and the media and

### Table 2: Effect of exposure to 10 ppm NO2 on incorporation of radioactivity into lamellar bodies of type II pneumocytes

<table>
<thead>
<tr>
<th>Cellular radioactivity (dpm/mg protein)</th>
<th>Radioactivity incorporated into lamellar bodies (% of cellular radioactivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-SP-A 13324 (1834) 184800 (9482) +SP-A 9.8 (12) 29.8 (2.1)</td>
</tr>
<tr>
<td>3 days</td>
<td>121968 (5702)** 264264 (12066)** -SP-A 16.3 (2.5)†† 35.9 (3.6)††</td>
</tr>
<tr>
<td>20 days</td>
<td>114576 (5948)** 260568 (12345)** +SP-A 14.8 (2.1)†† 34.4 (2.5)††</td>
</tr>
<tr>
<td>28 days</td>
<td>146916 (6481)** 305103 (14193)** -SP-A 16.6 (2.8)†† 34.9 (2.4)††</td>
</tr>
</tbody>
</table>

Values are mean (SE); n=3-4 experiments.

* p<0.01 between radioactivity from cells of different experimental protocols.

† p<0.05, †† p<0.01 between the percentage of radioactivity in the lamellar bodies from cells of different experimental protocols.

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**Figure 4** Time course of resecretion of ‘H-DPPC labelled liposomes from isolated type II cells from control animals and animals exposed for 3 days to 10 ppm NO2. The data represent the intracellular label as a percentage of the internalised material. Values are mean (SE), n=3-5; *p<0.05, **p<0.01 difference between time points of the 3 day exposure to NO2 and corresponding time points of control in the absence and presence of SP-A.

**Figure 5** Resecretion of internalised lipids from isolated type II cells from control animals and animals exposed to 10 ppm NO2 for 3, 20, and 28 days. The white bars represent remaining intracellular lipids from liposomes that have been internalised in the absence of SP-A, the black bars represent remaining intracellular lipids from liposomes that have been internalised in the presence of SP-A. The data represent the remaining intracellular label after lipid resecretion. Values are mean (SE), n=3-5; *p<0.05, **p<0.01 difference between time points of the 3 day exposure to NO2 and corresponding time points of control in the absence and presence of SP-A.

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Surfactant recycling in injured type II pneumocytes
In the absence of SP-A, the percentage of label to increase with the duration of NO2 exposure. This increase in PC label was significant for cells exposed to NO2 for 20 and 28 days. Label from the DPPC liposomes taken up by the SP-A mediated pathway was also found in the non-PC lipid moiety. Resecretion of lipid from cells from control and from NO2 exposed lungs incorporated a greater amount of label than cells from control lungs. After lipid resecretion, most of the remaining intracellular label stayed with the PC but most of the extracellular label was found in the non-PC lipid moiety. Resecretion of lipid from SP-A mediated liposome uptake resulted in an unmodified distribution of label from internalised ^3H-DPPC liposomes taken up by the SP-A mediated pathway was also incorporated into the PC moiety, but to a greater extent than in assays without SP-A. In these experiments, type II cells from different stages of lung injury are able to internalise and resecrete surfactant lipids. However, confocal laser scanning microscopy techniques it was confirmed that the data obtained from our assays were from intact/viable cells rather than from degenerating cells.

**DISCUSSION**

There is evidence from a number of studies that type II pneumocytes in normal lungs represent the major compartment for surfactant clearance from the alveolar space. However, there is no information on this process in diseased lungs. This mechanism is of interest for the therapeutic application of surfactant in injured lungs where it should be internalised by type II cells. The aim of the study was therefore to elucidate the extent to which type II cells from different stages of lung injury are able to internalise and resecrete surfactant lipids.

To experimentally induce lung injury, rats were exposed over different time periods to NO2. This procedure was found to generate different stages of lung injury showing histological correlates with acute and chronic bronchiolitis (after 3 and 20 days),

| Table 3 Fate of internalised and resecreted ^3H-DPPC containing liposomes |
|-----------------|-----------------|-----------------|-----------------|
|                  | 20 min internalisation | 20 min resecretion |
|                  | % intracellular label | % intracellular label | % extracellular label |
| NO2 exposure     |                  |                  |                  |
| 0 days           |                  |                  |                  |
| PC               | 80.3 [3.2]††      | 61.1 [2.4]       | 78.6 [3.7]       |
| Other lipids     | 18.7 [2.9]        | 38.9 [3.1]       | 21.4 [4.2]       |
| 3 days           |                  |                  |                  |
| PC               | 87.0 [3.7]††      | 59.8 [4.1]       | 67.6 [3.2]       |
| Other lipids     | 12.3 [3.1]        | 40.1 [3.9]       | 32.3 [5.3]       |
| 20 days          |                  |                  |                  |
| PC               | 88.4 [2.5]††      | 56.7 [2.0]       | 66.5 [2.9]       |
| Other lipids     | 11.5 [0.8]        | 43.3 [1.6]       | 34.4 [4.1]       |
| 28 days          |                  |                  |                  |
| PC               | 90.1 [2.2]††      | 55.4 [2.6]       | 68.1 [5.1]       |
| Other lipids     | 9.7 [0.8]         | 44.6 [1.8]       | 31.9 [4.8]       |
| In the presence of SP-A | 0 days          |                  |                  |
| PC               | 89.7 [1.4]**      | 87.1 [3.6]       | 92.0 [1.4]       |
| Other lipids     | 10.1 [1.9]        | 12.0 [0.9]       | 8.1 [2.0]        |
| 3 days           |                  |                  |                  |
| PC               | 94.3 [1.0]**      | 89.7 [4.7]       | 91.8 [1.1]       |
| Other lipids     | 5.9 [2.2]         | 10.3 [0.9]       | 8.1 [1.2]        |
| 20 days          |                  |                  |                  |
| PC               | 94.9 [2.3]**      | 92.4 [2.5]       | 90.3 [4.1]       |
| Other lipids     | 4.8 [1.0]         | 7.6 [0.5]        | 9.7 [0.3]        |
| 28 days          |                  |                  |                  |
| PC               | 96.9 [2.1]**      | 88.1 [6.1]       | 93.2 [2.2]       |
| Other lipids     | 3.0 [0.6]         | 11.9 [5.2]       | 6.8 [0.9]        |

Values are mean (SE); n=3–4 experiments.

††p<0.01 between label in the PC fraction after 20 minutes internalisation and 20 minutes resecretion.

**p<0.01 between all time points of liposome internalisation in the presence of SP-A and the corresponding time point in the absence of SP-A.

Values are mean (SE); n=3–4 experiments.

17 Apart from use of an appropriate uptake assay, it was necessary to determine whether lipid exchange and energy dependent internalisation in the absence and presence of SP-A contribute to the cell associated label. Type II cells from control and NO2 exposed lungs were incubated with cholesterol ester, a lipid marker that does not exchange easily, and it was found that, in the absence of SP-A, a certain amount of lipid results either from lipid exchange or from adherence to the cell membrane. This process does not require energy, as shown by the high association of cholesterol ester at 4°C. However, in the presence of SP-A, type II cells actively internalise a considerably larger amount of lipid. This was energy dependent because low temperature (4°C) reduced the SP-A mediated lipid uptake to the level found for unstimulated uptake (Table 1). This effect was observed for cells from both control and NO2 exposed lungs. It is clear that lipid uptake in these cells is composed of an active and a passive part. From this and results reported by other groups, it appears that the energy dependent SP-A mediated uptake contributes most of the lipid uptake. The different stages of
NO2 induced lung injury did not influence the relation between active and passive lipid uptake. In addition, accumulation of radioactivity in the lamellar bodies indicated lipid internalisation rather than simply adherence to the cell membrane.

As found in the kinetic experiments of liposome uptake (fig 1), there is a faster uptake into the cells from NO2 exposed lungs than in those from controls. The finding that there is no difference in the uptake kinetics between the cells from the different NO2 exposure groups suggests that the maximal effect was achieved by 3 days and could not be increased further by 20 and 28 days of exposure. The basis for this increase, however, is not known, but it is possible that the increased internalisation in NO2 exposed lungs is used for phospholipid synthesis. For type II cells from acutely NO2 injured lungs, an increased uptake and incorporation of choline into phospholipid was observed based on an increase in the specific activity of the choline kinase that showed increased phospholipid synthesis.

Although in vitro experiments have suggested that a number of functions in lipid metabolism have been directed to SP-A, including the stimulation of lipid uptake,21 in vivo the situation is different because a number of SP-A attributed functions in the lungs could not be found in vivo in SP-A gene targeted mice.22 In later life, however, the size of the surfactant pool of wildtype and knock out mice is no longer different.

As shown in fig 2, type II cells from all groups of NO2 exposed lung show increased liposome uptake compared with control cells. In these figures we preferred the calculation of lipid per cells rather than per protein because it is known that cell protein varies in cells. The fact that this increase was similar in the cells of all exposed lungs suggests that NO2 exposure may have disrupted cell membranes to allow liposome to enter the cells easily. However, LDH and trypan dye exclusion measurements before and after the washing procedures and incubation periods showed no cell degeneration. We therefore conclude that a mechanism is activated by the injury which leads to accumulation of lipid within the cells of NO2 exposed lungs. This internalisation could be used to take up pharmacologically active substances given to treat lung diseases, enabling the cells to direct the lipid material to intracellular pools for resecretion on demand and/or via multivesicular bodies and lysosomes to be used for de novo synthesis of surfactant phospholipid. This hypothesis is supported by other studies showing increased phospholipid synthesis in type II cells of diseased lungs.12 12 Moreover, if liposomes carrying drugs for treatment of lung injury were internalised, this would enable the lung to be treated directly at the place of impairment.

In summary, our study has shown for the first time that liposome uptake in type II cells from diseased lung is increased, allowing the lung to form intracellular phospholipid pools that enable it to cope with stress situations such as lung injury. The reduced resecretion does not argue against this possibility, because our experimental approach only analysed the in vitro situation and did not include factors that stimulate surfactant phospholipid secretion in vivo in diseased lungs. Further studies of the mechanisms involved in surfactant activity are necessary to understand better the basic mechanisms of surfactant metabolism in type II cells of diseased lungs to develop treatment strategies for lung diseases such as COPD.

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