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Breathing and pulmonary surfactant function in mice 24 h after ozone exposure

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Breathing and pulmonary surfactant function in mice 24 h after ozone exposure. W.D. Currie, S. van Schaik, I. Vargas, G. Enhorning. ©ERS Journals Ltd 1998.

ABSTRACT: The aim of this study was to determine whether an acute ozone exposure affects breathing, and the ability of pulmonary surfactant to maintain the patency of terminal conducting airways.

BALB/c mice were exposed to ozone (1 part per million (ppm)) for 2, 4, 6, and 8 h. They were examined with plethysmography and with bronchoalveolar lavage (BAL) 24 h later. The BAL fluid was analysed for the presence of inflammatory cells and concentrations of proteins and phospholipids. Surfactant in the remaining BAL fluid was concentrated five-times and examined with a capillary surfactometer (CS). The surfactant was then washed with a large volume of saline solution which was removed following centrifugation.

Already, after a 2 h ozone exposure, the respiratory frequency increased from 297±6 to 386±11 breaths·min^{-1} ($p<0.0001$). Pressure amplitude per breath diminished ($p<0.001$), indicating a reduced tidal volume. A highly significant surfactant dysfunction was observed with the CS ($p<0.0001$), although phospholipids increased. However, proteins also increased ($p<0.0001$) and they or other water-soluble inhibitors apparently caused the surfactant dysfunction since, when they were removed with a washing procedure, the surfactant's normal ability to maintain patency was restored.

The acute ozone exposure affected breathing and caused an airway inflammation. The inflammatory proteins or other water-soluble inhibitors reduced the surfactant's ability to secure airway patency.

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A recent report showed that the ubiquitous air pollutant ozone affects breathing and pulmonary surfactant function in BALB/c mice [1]. The animals had been exposed to a relatively high concentration of ozone (2 parts per million (ppm)) for 2, 4, 6 and 8 h and they were examined immediately after they had been removed from the ozone environment. Their breathing, studied with a whole-body plethysmograph, was clearly affected, but they appeared to be tranquilized and, therefore, it was difficult to determine whether the breathing was altered because of an effect on the central nervous system (CNS), or because the airways had been affected. However, the surfactant function of the bronchoalveolar lavage (BAL) fluid was increasingly disrupted with increasing duration of ozone exposure. This was suggested to be due to surfactant inhibitors, such as plasma proteins and inflammatory cells, which appeared in BAL fluid in greater amounts with increasing duration of ozone exposure. Presumably, the inhibiting effect was partly due to a considerable leakage of plasma proteins into the airway lumen.

The inflammatory changes observed in the BAL fluid were so substantial that they would probably also be noted with a lower ozone dose and remain present even if the animals were not examined until later, when the effect on the CNS had vanished. Therefore, the present study was initiated. The mice were exposed to ozone for the same durations but to a lower concentration (1 ppm) and they were not examined until 24 h after they had been removed from the ozone environment. Their breathing was again evaluated with a whole-body plethysmograph and the BAL fluid was analysed with regard to surface properties, inflammatory cells and concentrations of proteins and phospholipids.

Materials and methods

Animals

A total of 96 male BALB/c mice, 7 weeks old and weighing 21–29 g, was studied. Four groups of 16 animals were exposed to 1 ppm ozone for 2, 4, 6 and 8 h respectively. To serve as controls, 32 mice were caged under identical conditions but breathed only filtered room air. The study was approved by the Institutional Animal Care and Use Committee.

Ozone exposure

The animals were housed in individual wire-mesh modules placed in a 0.3 m^3 Rochester type chamber [2]. The ozone, generated from O_2 using a silent-arc discharge O_3

generator (OREC, Phoenix, AZ, USA), was mixed with filtered room air and introduced into the chamber at a rate of one chamber volume·min^{-1}. The concentration of O_3 was monitored continuously with chemiluminescent O_3 analysers (Bendix, Louisbourg, WV, USA). Chamber concentrations of O_3 in these studies deviated <3% from the intended concentration of 1 ppm. The relative humidity in the chambers was maintained at 40–70% and the temperature between 18 and 26°C.

Plethysmography

Twenty-four hours after the animals had been removed from the ozone exposure they were studied with a whole-body plethysmograph. They could, thus, be examined without being anaesthetized or restrained. The design of the particular plethysmograph used for this study has been reported previously [1, 3]. It is characterized by the small size of the chamber housing the animal. Its volume is only 80 mL and therefore there has to be a continuous flow of fresh air through the chamber, which is interrupted only during the short periods of recording. Chamber pressure was measured with a transducer (Grass PT5) and recorded with an AstroMed recorder model 7400 (AstroMed, West Warwick, RI, USA). From the record of the animal's breathing it was possible to deduce the respiratory frequency (f_R), the mean of the pressure change caused by inspiration and expiration (ΔP), and the ratio between the duration of inspiration and the duration of expiration (t_I/t_E).

Bronchoalveolar lavage

Following the study of breathing, a lethal dose of pentobarbital (100 mg·kg^{-1}) was injected *i.p.* and BAL was performed. An 18-gauge metal tube was inserted into the trachea and tightly secured. A volume of 150 mM saline solution, corresponding to 2% of the animal's body weight, was used for the lavage procedure. It was carried out with a pressure- rather than a volume-controlled technique [3]. When pressure of the solution was raised to 30 cmH_2O, the liquid moved into the airways, whereby the lungs became expanded, evenly and gently. As the liquid was withdrawn again, with a negative pressure of 20 cmH_2O, tiny air bubbles appeared. They floated up and formed a layer of foam. To give the BAL fluid a high concentration, the lavage procedure was repeated six times with the same liquid. The foam floating on top of the BAL fluid was rich in pulmonary surfactant and it was liquefied to achieve an even dispersion of the foam material. The BAL fluid was repeatedly exposed to a negative pressure of 80 kPa, which caused the bubbles to expand until they burst and, in this way, the wall material of the bubbles was suspended in the liquid.

Surfactant evaluation

After aliquots of the BAL fluid, required for an assessment of proteins, phospholipids and cells, had been removed, the remaining fluid was centrifuged at 40,000×g for 1 h at 4°C. A volume of supernatant equal to 80% of the centrifuged BAL fluid was removed and the surfactant in the pellet was resuspended in the remaining 20% of the fluid. This raised the surfactant concentration approximately five-fold.

A capillary surfactometer (CS) was used for an assessment of the BAL fluid's ability to maintain patency. This instrument, described previously [4–6], uses a narrow glass capillary as a model of a terminal conducting airway. The capillary has a constricted area where the width (radius 0.1 mm) is similar to that of the terminal human airway [7]. A small volume of the liquid to be evaluated (0.5 μL) is deposited in the constricted area. Owing to surface tension the liquid remains in this area, forming a column that blocks the capillary lumen. Pressure is continuously recorded as it is slowly raised on one side of the liquid column. Eventually, the liquid is extruded from the narrow section. If it contains properly functioning pulmonary surfactant it will not return to the narrow section and patency will thereby be secured. Air continues to flow through the capillary without meeting resistance, whereby pressure remains at zero. If the surfactant is inadequate in quantity or function, the liquid will return to the narrow section of the capillary. Free airflow will be prevented and pressure will not remain at zero, but will increase repeatedly. The capillary thus simulates the physical conditions of the terminal conducting airway. The airflow is maintained for 120 s after the liquid was first extruded and the percentage of the total study time for which the capillary was open is calculated ("open in %") by the CS. The "open in %" value for a mouse was the mean of 10 assays of its concentrated BAL fluid.

Evaluation of surfactant inhibitors

To investigate the possibility that the surfactant function was inhibited by water-soluble agents, the surfactant in the BAL fluid was washed with the following technique. To the 20% of the BAL fluid remaining after the first centrifugation for 1 h, a relatively large volume (500 μL) of a 150 mM saline solution was added. The total fluid was vortexed and then centrifuged at 40,000×g for another hour. Of the supernatant, 500 μL was removed, a volume equal to that added previously. The remaining liquid, approximately 50 μL, containing the washed pulmonary surfactant, was vortexed and studied with the CS.

Protein and phospholipid analyses

The method of Lowry *et al.* [8] was used for an analysis of BAL fluid protein concentration. Phospholipids were extracted according to Bligh and Dyer [9] and the phosphorus content was determined with the micromethod of Chen *et al.* [10]. In selected cases the phospholipid composition was evaluated with thin-layer chromatography (TLC) using the solvent system of Touchstone *et al.* [11].

Evaluation of cells in bronchoalveolar lavage fluid

The cells in a portion (50 μL) of the original lavage fluid were centrifuged on a microscope slide using a cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA). The cells were fixed and stained with Wright-Giemsa

(CMS, Houston, TX, USA). A differential count of 200 cells·slide^{-1} was carried out under light microscopy. Based on morphological and staining properties each cell was assigned to one of three groups: mononuclear phagocytes, lymphocytes, or neutrophilic granulocytes.

Statistics

Results are expressed as means±SEM. A Student's t-test was used for the comparison of two groups. When data were not normally distributed, a Mann-Whitney rank sum test was carried out. One-way analysis of variance (ANOVA) on ranks according to a Kruskal-Wallis rank sum test was performed. A significant difference was assumed to exist when $p<0.05$.

Results

Twenty-four hours after the animals were removed from the ozone environment all of them were alive and none showed drowsiness or any obvious sign of morbidity. There was no conspicuous difference between the mice exposed to ozone and the controls.

Breathing patterns

Figure 1 shows typical examples of tracings showing how the breathing pattern, evaluated with the whole-body plethysmograph, changed as the duration of ozone exposure was prolonged: f_R increased, ΔP diminished and, after 6 and 8 h, t_I/t_E was reduced, mainly because expiration required more time. Figure 2a shows how the parameter that was most readily evaluated, f_R, was affected. The 32 animals never exposed to ozone (the 0 h group) had a f_R of 297±6 breaths·min^{-1}. In the group of mice exposed to ozone for only 2 h, f_R had already increased significantly, to 386±11 breaths·min^{-1} ($p<0.0001$), but with longer durations of ozone exposure there was no further increase in f_R.

As shown in figure 2b, ΔP diminished with ozone exposure ($p<0.001$), which probably indicated a reduced tidal volume. In addition, t_I/t_E showed a significant reduction after 6 and 8 h of ozone exposure ($p<0.01$, fig. 2c).

Surfactant function

In the control animals (the 0 h group) the "open in %" was 78±5, indicating that the BAL fluid generally had a good ability to maintain patency. However, the capability to preserve patency gradually diminished with duration of ozone exposure ($p<0.0001$) and after 8 h the "open in %" was only 16±2 (fig. 3). By washing the surfactant in the BAL fluid, by adding 0.5 mL saline solution to the concentrated BAL fluid, centrifuging and again removing the 0.5 mL added fluid, water-soluble inhibitors were separated from the surfactant. When the washed material was evaluated once again, under the same conditions as before the washing, the "open in %" increased dramatically, even in the 0 h group (open bars in fig. 3a, $p<0.0001$).

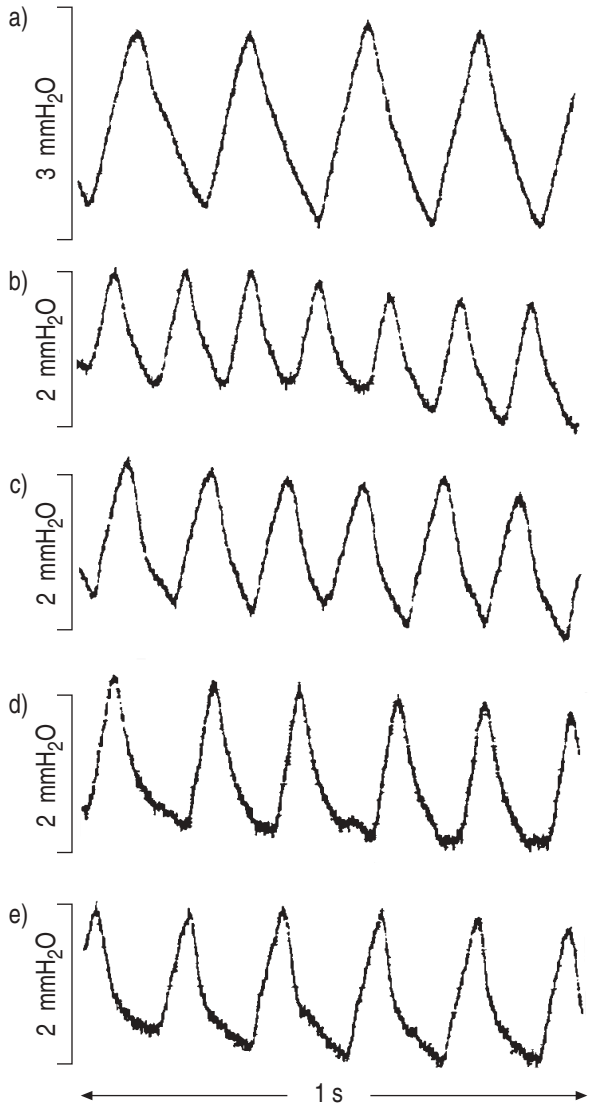

Fig. 1. – Typical tracings obtained with the whole-body plethysmograph of a) unexposed mice and mice exposed to 1 ppm ozone for b) 2; c) 4; d) 6 and e) 8 h. The respiratory frequency increased. Pressure change during breathing, ΔP, diminished. The ratio between inspiration and expiration time was reduced after 6 and 8 h. The increase in pressure, indicating inspiration, is distinct.

Protein concentration of bronchoalveolar lavage fluid

The protein concentration of the BAL fluid increased as the ability of the fluid to maintain patency decreased (fig. 3b). The protein concentration in the control (0 h group) was 0.45±0.04 mg·mL^{-1}. It gradually increased and in the 8 h group it was 1.46±0.14 mg·mL^{-1}, a change that was highly significant ($p<0.0001$).

Phospholipid concentration of bronchoalveolar lavage fluid

The phospholipid concentration in the control (0 h group) was 0.35±0.02 mg·mL^{-1} and, starting in the 4 h group, it increased and reached a concentration of 0.57±0.05 mg·mL^{-1} in the 8 h group ($p<0.01$, fig. 4).

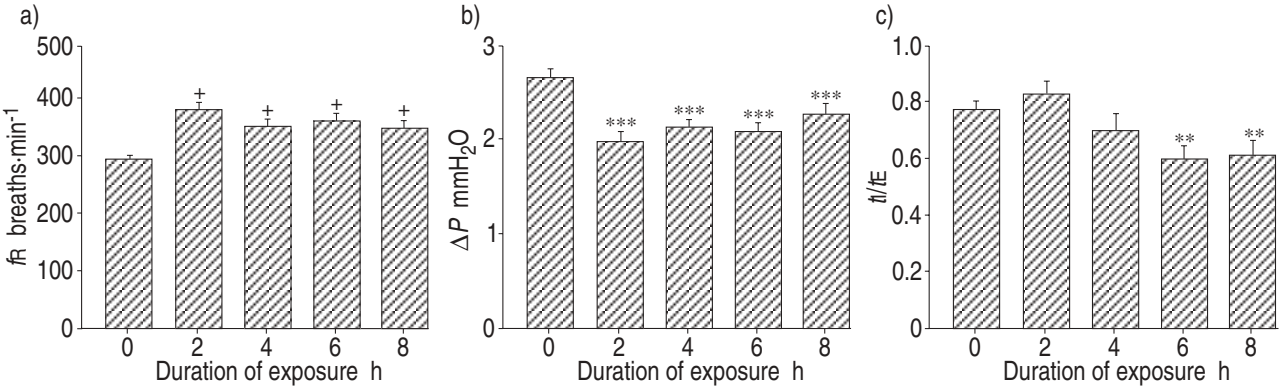

Fig. 2. – Results obtained from studies with the plethysmograph. a) All animals exposed to ozone had a clearly raised respiratory frequency (*f*R). +: p<0.0001 compared with the control (0 h group). b) The pressure change (ΔP) exerted with each breath diminished after 2 h of ozone exposure. Under the conditions of this study it indicated a reduced tidal volume. ***: p<0.001 compared with the control (0 h group). c) The ratio between duration of inspiration and expiration (*t*I/*t*E) was significantly diminished after 6 and 8 h. **: p<0.01 compared with the control (0 h group).

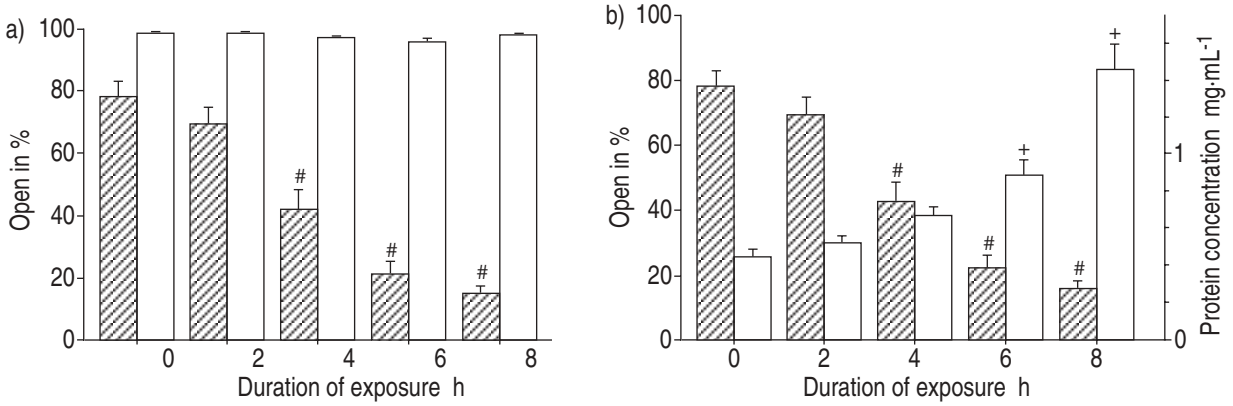

Fig. 3. – a) The bronchoalveolar lavage (BAL) fluid's ability to maintain patency (▨) gradually diminished with the duration of ozone exposure. #: p<0.0001 compared with the control (0 h group). However, following the removal of water-soluble inhibitors, the surfactant's ability to maintain patency became optimal (□). b) The BAL fluid's ability to maintain patency (▨) diminished (p<0.0001) as the fluid's protein concentration (□) increased. +: p<0.0001 compared with the control (0 h group). Removal of the proteins with the washing procedure was probably a major reason for the improvement observed in a).

Bronchoalveolar lavage fluid cells

The percentage of neutrophilic granulocytes slowly increased with the duration of ozone exposure and at 6 and 8 h their proportion had increased significantly (p<0.0001) (fig. 5).

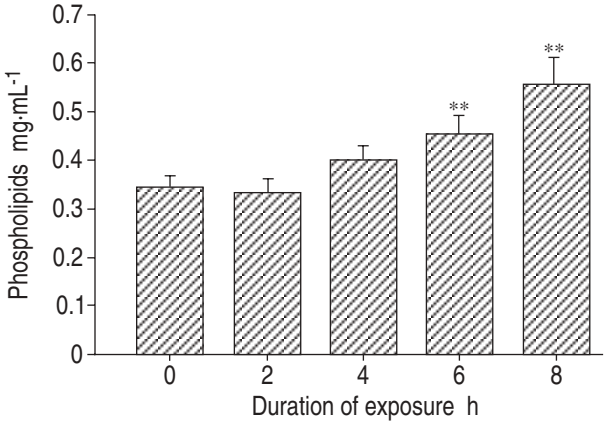

Fig. 4. – Mice exposed to ozone for 6 h or more showed a significant increase in the concentration of phospholipids. **: p<0.01 compared with the control (0 h group).

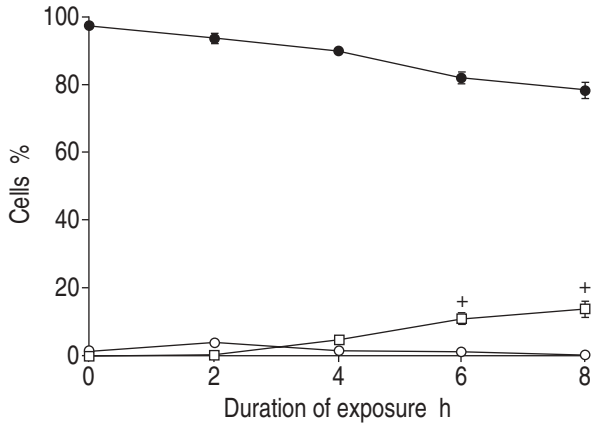

Fig. 5. – With increasing duration of ozone exposure the percentage of neutrophilic granulocytes increased significantly. +: p<0.0001 compared with the control (0 h group). ● : mononuclear phagocytes; ○ : lymphocytes; □ : neutrophilic granulocytes.

Discussion

Respiration

In a previous study, when mice had been exposed to a high dose of ozone (2 ppm) and examined shortly after the

exposure, their f_R was depressed [1, 12]. This was probably due to a direct effect of the gas on the CNS since it was not observed in the present study.

The increased f_R observed in this study was possibly due to hypercapnia developing as a result of poor gas exchange, which in turn might have been caused by a blockage of several conducting airways so that air in the corresponding alveoli was trapped or at least inadequately replaced during breathing. BAL clearly verified the presence of inflammation in the airways. Consequently, there probably was oedema in the airway wall and surrounding tissue, which diminished the airway lumen and increased airway resistance. An augmented resistance to airflow would also be caused if pulmonary surfactant had lost some of its ability to preserve airway patency. Indeed, there was such a loss of surfactant ability, which would probably be a reason for the formation of liquid columns blocking the airway lumen. This would affect the most narrow airways, particularly if they had become narrower owing to an inflammatory reaction [5] and when, at end expiration, their lumen had been reduced even further. During inspiration, the blocking liquid may be moving into alveoli where it could be absorbed. If not, it may be extruded again into the conducting airways with the next expiration. Once again, it may then form blocking liquid columns at end expiration.

It is also conceivable that oedema in the airway wall caused a stimulation of Paintal's juxtaalveolar receptors (j-receptors) [13] and this may also have contributed to the increased f_R.

Following ozone exposure, f_R increased, whereas ΔP was reduced. With a mechanical chest it has been shown that a reduction in ΔP can occur not only because of a reduced tidal volume but also as a result of a lower respiratory rate and/or a reduced airway resistance (Enhorning *et al*., unpublished observations). Since, under the conditions of the present study, f_R was increased and, in all likelihood, airway resistance was increased, it can be deduced that the observed lowering of the value of ΔP indicated that tidal volume had been reduced.

A reduction in t_I/t_E was seen following 6 and 8 h of ozone exposure (figs. 1 and 2c), partly because inspiration became faster, but there was also a prolongation of the time required for expiration. As shown in figure 1 the inspiration caused a steep increase in plethysmograph pressure and the beginning and the end of the pressure increase were quite distinct. However, in some breaths it was difficult to determine whether the minimal pressure change preceding an inspiration was the beginning of the inspiration or the end of the preceding expiration. Such an ambivalent period was defined as belonging to the ex-piration. After prolonged ozone exposure, *i.e*. 6 and 8 h, the end of expiration was protracted, perhaps indicating that expiration was meeting a substantial resistance.

Surface properties of bronchoalveolar lavage fluids

The surface properties of the BAL fluids gradually deteriorated with the duration of ozone exposure (fig. 3). The surfactant of the fluid was less capable of securing patency. Since the phospholipid concentration increased (fig. 4) and thin-layer chromatography gave no obvious indication that the composition of phospholipids had been changed, it appeared more likely that the poor ability of the surfactant to maintain patency was due to inhibition. That supposition was strongly supported when it was found that the ability to maintain patency came very close to 100% when the surfactant had been washed (fig. 3a). It is conceivable that the proteins that had leaked in increasing quantities into the airway with lengthening ozone exposure, had been washed away and that they had been the main reason for the inhibition (fig. 3b). These observations that exposure to ozone causes a loss of surface activity due to a protein inhibition are in line with a recent publication by PUTMAN *et al*. [14]. These authors found that ozone exposure reduced the activity of pulmonary surfactant. They felt this was due to an inhibiting effect of inflammatory proteins. However, they also noticed that the extracted surfactant had lost some of its activity, in that its adsorption was delayed. The extremely hydrophobic apoproteins surfactant protein (SP)-B and SP-C should have been extracted with the lipids but, since their adsorption promoting effect was not observed, the authors suspected that the apoproteins had been adversely affected by ozone exposure.

The observation in this study that the phospholipid concentration increased following exposure to ozone contrasted with the previous observation [1] that the concentration diminished. However, in the previous investigation, the phospholipid concentration was determined immediately after the ozone exposure, whereas in the present study there had been a 24 h recovery period. Considering this change in timing, these observations are not contradictory but are in line with previous studies showing that immediately after exposure to high, and therefore toxic, oxygen concentrations, the phospholipids diminished but later recovered [15]. These important questions of how ozone, by virtue of its oxygen toxicity, affects pulmonary surfactant have been reviewed recently [16].

The observation that there was a greater release of pulmonary surfactant concomitant with a higher f_R was also in accordance with the observations made by NICHOLAS and BARR [17] and POWER *et al*. [18]. They found that when rats were breathing more vigorously because they were forced to swim, more surfactant was released into the airways. The mice in the present experiments proba-bly also had to expend more energy on breathing since their f_R was higher and they likely had an increased airway resistance to overcome.

As the duration of ozone exposure increased, the surfactant in the BAL fluid gradually lost its ability to ensure patency. This indicated that the resistance to airflow would be greater the longer the duration of ozone exposure. Nevertheless, the values of f_R and ΔP gave no indication of ongoing deterioration; they changed after 2 h but no more after that time. However, it is conceivable that the reduced value of t_I/t_E observed after 6 and 8 h indicated a greater resistance to airflow at the end of expiration.

Although the inability of the BAL fluid to maintain patency correlates well with the mean protein concentration, the correlation was often weak in individual cases, particularly in the groups of mice exposed to ozone for only 2 or 4 h. This may relate to the type of plasma protein that had leaked into the airway. The major protein that is likely to invade the airway, albumin, is a relatively weak inhibitor, whereas fibrin is very potent [19, 20]. Future studies

are planned to concentrate on a careful analysis of proteins in BAL fluid. The aim will be to investigate the possible appearance of fibrinogen, fibrin, gamma-globulin or other inhibitors that are more potent than albumin.

The inflammatory reaction, which was so apparent 24 h after the ozone exposure, was concomitant with a deteriorating surfactant function. It was recently noted that the inflammation caused by an infection with respiratory syncytial virus was also associated with surfactant dysfunction [6]. One could speculate that any inflammatory reaction, including asthma, causing a leakage of plasma proteins into the airway lumen will be associated with surfactant dysfunction.

In conclusion, this study showed that respiratory frequency was increased 24 h after ozone exposure, although the time of expiration was prolonged. Furthermore, there were indications that tidal volume had been reduced. The bronchoalveolar lavage fluid showed signs of inflammation and the inflammatory proteins probably inhibited surfactant, so that it gradually lost its ability to preserve patency. However, that ability was recovered when the proteins and other water-soluble agents had been washed away.

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