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Lung Surfactant Components in Bronchoalveolar Lavage After Inhalation of NO_2 as Markers of Altered Surfactant Metabolism

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Abstract. To study the effects of nitrogen dioxide (NO_2) inhalation on lung lavage surfactant components as markers of an altered surfactant metabolism in type II pneumocytes, rats were exposed to atmospheres with increasing NO_2 concentrations (0.8, 5.0, and 10.0 ppm) over 1 and 3 days. After exposure lung lavage was performed and surfactant components as well as lavagable cells analyzed. An increased number of total lavage cells was found with increasing concentration and duration of NO_2 exposure. Cell distribution showed an elevation in the number of granulocytes and lymphocytes whereas the number of macrophages was diminished. The amount of total lavage protein revealed an increase related to NO_2 concentration and duration. Also the content of lavage phospholipid was increased, with a decreased portion of phosphatidylcholine (PC). Further analyses of PC showed a diminished composition of saturated fatty acids but an elevated content of the unsaturated portion. Functional studies on surfactant phospholipid extracts exhibited comparable values for the surface tension at equilibrium, as well as for the maximal and minimal surface tension of animals exposed to 0.8 ppm NO_2 and controls. Higher NO_2 concentrations (5 and 10 ppm) resulted in increased values for surface tension compared to controls. This was also observed with purified surfactant that was obtained from controls and from NO_2-exposed rats. These experiments show that in vitro exposure of purified surfactant to NO_2 atmospheres was more effective than exposure in vivo. When the structure of the surfactant proteins A was studied it was found not to be altered by the NO_2. The data clearly demonstrate that NO_2 inhalation impaired function of surfactant components that may be used as markers of altered surfactant metabolism.

Key words: Nitrogen dioxide—Bronchoalveolar lavage—Surfactant protein A (SP-A)—Phospholipids—Surface tension.

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Introduction

Of all atmospheric air pollutants, nitrogen dioxide (NO_2) is one of the major components and it has been shown in various experimental animal studies to produce lung injury [1, 3, 10, 18]. It exists as a free radical with limited aqueous solubility and its uptake within the lung is more related to chemical reactions than to reactions due to physical solution. Nitrogen dioxide undergoes quantitative, irreversible chemical reactions as a consequence of its pulmonary uptake, and these may occur near the airspace surface [22]. However, the precise mechanism of NO_2-induced lung toxicity is still unknown. Apart from histological alterations in the bronchoalveolar transition zone, it seems likely that the epithelial lining material containing components of the surfactant system as well as the cellular composition of the alveolar airspace may be altered by NO_2 exposure.

The present study was designed to evaluate dose related effects of in vivo exposure to NO_2 on several lung surfactant components obtained by bronchoalveolar lavage. Additionally, surfactant protein A (SP-A), regarded to be a modulator of the surfactant system, was evaluated for structural alteration by NO_2 exposure. These results could help to elucidate the interaction between type II pneumocytes and environmentally impaired SP-A. The NO_2 concentrations used in these studies ranged up to 10 ppm; although this is rather high as far as urban concentrations are concerned, there are circumstances under which NO_2 concentrations may be considerably higher. In inhaled cigarette smoke the concentration of NO_2 can be on the order of 250 ppm [8, 21]; also, in silo-filler's disease very high concentrations of this gas may occur [27]. The exposure model provides an insight into in vivo effects in the thermal lung after acute NO_2 exposure. The analysis of the epithelial lining material will contribute to understanding of the mechanisms of NO_2 toxicity within the alveoli, and will be complementary to other parameters that have been described for humans [7, 15, 17, 24] and laboratory animals [25, 28].

Materials and Methods

Materials

All reagents used were of analytical grade and purchased from Sigma (Deisenhofen, Germany) unless otherwise stated. The NO_2 was delivered in gas tanks (Messer Griesheim, Duisburg, Germany) and was adjusted to the desired concentration by dilution with compressed normal air.

NO_2 Exposure System

For all experiments, specific pathogen-free, male Sprague-Dawley rats (Charles River Wiga, Sulzfeld, Germany) with a body weight of 200–200 g were used. They were kept 4 animals to a cage with water and food *ad libitum*. The cages were placed into gas-tight chambers equipped with an inlet and outlet for the NO_2 gas mixture, a ventilator to produce equal distribution of the gas atmosphere throughout the boxes, as well as a sample port to allow measurements of the actual

NO_2 concentration. A weather station was used to control atmospheric pressure, humidity, and temperature. The volume of the exposure boxes was 60 liters and the gas flow was adjusted to 15 l/min. The humidity of the exposure atmospheres was kept at 50 ± 9%, and temperature at 24 ± 2°C. At the beginning of the experiments the actual gas concentration within the chambers was controlled every 2 hours, later a 6-h determination period was sufficient to guarantee a constant gas atmosphere. NO_2 concentrations were determined spectrophotometrically against standard solutions (made daily) according to the method of Saltzman [23]; a single gas concentration was the mean of two determinations taken from the middle of the chamber. The NO_2 concentrations used in these experiments were 0.8, 5.0, and 10 ppm; exposure periods were 1 and 3 days. As a control, rats were exposed in the same way to compressed normal air.

BAL Procedure

To obtain bronchoalveolar lavages the rats were anaesthetized with pentobarbital sodium (50 mg/kg) that was mixed with 1000 IU of heparin sodium and injected intraperitoneally. When the animals were under deep anaesthesia the trachea was cannulated and the lungs were removed from the body after thoracotomy. Lung lavage was performed extracorporally with 4 volumes of 8 ml of normal warm (37°C) saline. After each lavage of the fluid was allowed to run out passively. The bronchoalveolar lavage fluid was centrifuged at 300*g* and 4°C for 10 min and the pellet was analyzed to determine the cell population composition as described below.

Lavage Cells

For microscopical analyses, cells were treated as cytospin spreads, and stained with hematoxilin-eosin and Schiff's perjodic stain. For each rat lavage, the total cell number and the distribution of macrophages, granulocytes, and lymphocytes were determined.

Phospholipid Analyses

From the alveolar wash, aliquots were extracted for phospholipid analyses according the the procedure described by Folch et al. [11]. After extraction from the lavage fluid, the phospholipid classes were analyzed by thin layer chromatography on silica H-60 plates (Merck, Darmstadt, Germany). The solvent was methanol : chloroform : benzene : acetic acid : boric acid (20 : 40 : 30.10 : 1.8g; v/v/v/v/w), and after the separation the phospholipid bands were visualized with iodine vapor. For quantification, the bands were scraped off from the plates and the lipid phosphorous measured [2].

Determination of the fatty acid content in the band of the phosphatidylcholines was performed after hydrolysis and methylation [26], followed by analysis on a model 419 gas chromatograph with a model 3380 A integrator (both Hewlett-Packard, Frankfurt, Germany). The separation was performed at 180°C on an EGSSX column (Serva, Heidelberg, Germany). The contents of saturated and unsaturated fatty acids in the phosphatidylcholines was calculated from the gas chromatographic analyses [H. H. Schäfer (1990) Thesis. Phillipps University, Marburg, Germany].

Surface Tension Measurements

The ability of phospholipids to spread and to lower surface tension at the air/water interface was studied on a Wilhelmy balance (Biegler, Mauersbach, Austria). These experiments were performed at 37°C with identical amounts of extracted surfactant phospholipids with a concentration of 15 μg/ml (final concentration at the air/water interface was 13.6 μg phospholipid per cm^2). Surface

adsorption was recorded over a period of 60 min to insure equilibrium surface tension. To determine the maximal and minimal surface tension, cyclic compression of the phospholipid film at the liquid/air interface was performed; the cycle speed was adjusted to 1.5 min/cycle and the compression area was 20% of the surface (total surface was 22 cm^2). The measurements were terminated when the percentage in the difference in values of the last three cycles was smaller than 1%. To evaluate the suitability of lung lavage extracts for surface tension measurements, a comparison to purified rat surfactant from normal and exposed (10 ppm NO_2, 3 days) animals was performed. For this purpose rat surfactant was purified according to the procedure of Katyal and coworkers [16].

Preparation of SP-A

SP-A was extracted with the butanol method according to Hawgood et al. [13] from lavages that were either from controls or from rats exposed to 10 ppm NO_2 for 3 days. Both types of SP-A preparations were tested for structural differences in immunoblot analyses after gel electrophoresis as described elsewhere [20].

Histology

After killing and exsanguination, the thorax was opened and the mediastinum and the lungs were excised in toto. One lung was filled intrabronchially with 4% formaldehyde solution at a pressure of 20 cm H_2O. The bronchus was clamped and the lung was immersed in a 4% formaldehyde solution where it was left floating freely for at least 48 h. After fixation the lung was cut in the frontal plane. Tissues were dehydrated in ethanol and embedded in parraffin. For histological and morphometrical investigations, 5 μm sections, stained with hematoxilin-eosin (HE) were prepared.

Related Procedures

Protein determinations were carried out according to Bradford [6] using bovine serum albumin as a standard, with the reagents obtained from Biorad (München, Germany). The quantification of phospholipids was performed by determining the phosphorus content according to the method of Bartlett [2]. Denaturing gel electrophoresis was performed using the Excelge system with a continuous polyacrylamide gradient ranging from 8 to 20% (w/v) according to the instructions of Pharmacia (Freiburg, Germany).

Statistics

Significance of the results was calculated with the analysis of variance test on an IBM personal computer using the Statgraphics program (Statistical Graphics, Rockville, Maryland). Values were expressed as mean ± SD. The results were considered significant with an error less than or equal to $P = 0.05$.

Results

Cellular Components of the Bronchoalveolar Lavage

When the bronchoalveolar lavage was performed extracorporally, a recovery rate was obtained that did not differ between exposed and control rats (95.4 ± 1.2%, n = 144). Lavagable cells from the terminal lung increased in number

Table 1. Influence of in vivo exposure to NO_2 on components of rat bronchoalveolar lavage

Treatment	(n)	Cells ($\times 10^6$/rat)	Protein (mg/rat)	Phospholipid (mg/rat)
Control	(36)	2.6 ± 1.7	3.2 ± 1.1	0.8 ± 0.4
0.8 ppm/1 day	(18)	2.8 ± 1.9	3.9 ± 0.7	0.8 ± 0.3
3 days	(18)	2.4 ± 0.8	3.5 ± 1.0	0.7 ± 0.5
5.0 ppm/1 day	(18)	$3.8 \pm 2.2^{*}$	3.9 ± 0.8	0.8 ± 0.3
3 days	(18)	$4.0 \pm 1.4^{*}$	$4.8 \pm 0.7^{*}$	0.9 ± 0.3
10.0 ppm/1 day	(18)	$4.3 \pm 1.8^{*}$	$7.6 \pm 3.8^{*}$	$1.4 \pm 0.2^{*}$
3 days	(18)	$14.5 \pm 5.6^{**,++}$	$6.6 \pm 1.5^{**,++}$	$1.5 \pm 0.6^{*}$

The results are expressed as mean $\pm$ SD, n = number of animals; being different from control with * = $P \leq 0.05$ and ** = $P \leq 0.01$ and being different from the corresponding exposure to 24 hour with $^{++}$ = $P \leq 0.01$

Table 2. Distribution of cell populations in the lavages from NO_2-exposed rats

Treatment	(n)	Macrophages	Granulocytes	Lymphocytes
Control	(16)	98.6 ± 0.8	0	1.4 ± 0.4
0.8 ppm/1 day	(12)	99.0 ± 0.6	0	1.0 ± 0.2
3 days	(12)	$98.3 \pm 1.0^{+}$	0	1.7 ± 0.2
5.0 ppm/1 day	(12)	98.8 ± 1.1	0.4 ± 0.4	0.8 ± 0.4
3 days	(12)	98.8 ± 1.6	0.2 ± 0.2	1.0 ± 0.8
10.0 ppm/1 day	(12)	$94.8 \pm 2.1^{**}$	$3.4 \pm 1.3^{**}$	1.8 ± 0.8
3 days	(12)	$87.0 \pm 2.4^{**,++}$	$11.4 \pm 2.9^{**}$	1.6 ± 1.2

The cell populations are expressed as percentage of total cells and represent mean $\pm$ SD; n = number of animals; being different from control with ** = $P \leq 0.01$ and being different from the corresponding exposure to 24 hours with $^{+}$ = $P \leq 0.05$ and $^{++}$ = $P \leq 0.01$

after NO_2 exposure depending on the gas concentration and the time of exposure (Table 1). With increasing concentration and duration of NO_2 the content of macrophages decreased, whereas the percentage of granulocytes and lymphocytes increased (Table 2).

Phospholipid Component and Surface Activity

In the bronchoalveolar lavage the amount of phospholipid revealed a dose and exposure time dependence. Whereas exposure to 0.8 ppm and 5 ppm NO_2 resulted in no difference for the total washed out phospholipid, an increase in phospholipid was observed after exposure to 10 ppm NO_2 (Table 1). Phospholipid composition analyes of lavages from control and NO_2-exposed animals showed no major differences except for a slightly decreased phosphatidylcholine

Table 3. Content of the phosphatidylcholine in bronchoalveolar lavage phospholipids after in vivo exposure to NO_2

Treatment	(n)[a]	PC[b] (%)	sat PC[c] (%)	unsat PC[c] (%)	16:0 of PC[d] (%)	20:4 of PC[d] (%)
Control	(32)	75.9 ± 6.1	91.4 ± 4.3	5.6 ± 2.7	84.0 ± 4.6	0.8 ± 1.1
0.8 ppm/1 day	(12)	73.4 ± 4.5	93.3 ± 3.2	5.0 ± 2.9	84.9 ± 4.8	0.8 ± 1.2
3 days	(12)	73.0 ± 7.8	94.1 ± 2.3	3.5 ± 2.1	84.0 ± 2.2	0.8 ± 1.2
5.0 ppm/1 day	(12)	73.5 ± 7.3	90.4 ± 3.8	4.0 ± 3.0	85.3 ± 4.2	0.3 ± 0.6
3 days	(12)	73.0 ± 8.0	87.1 ± 7.4	8.2 ± 3.5++	84.0 ± 2.8	1.3 ± 1.4
10.0 ppm/1 day	(12)	73.9 ± 7.7	81.6 ± 7.4**	11.2 ± 2.9**	77.1 ± 3.6**	4.5 ± 2.8**
3 days	(12)	71.6 ± 9.4	85.1 ± 3.8**	12.1 ± 3.6**	73.0 ± 8.2**	4.0 ± 2.4**

[a] n, number of animals
[b] percentage of lavage phosphatidylcholine (PC)
[c] percentage of saturated (sat) and unsaturated (unsat) phosphatidylcholine
[d] 16:, palmitic acid; 20:4, arachidonic acid
the results are as percentage and represent mean ± SD; being different from control with ** = $P \leq 0.01$ and being different from the corresponding exposure to 24 hours with ++ = $P \leq 0.01$

(PC) content in the exposed animals (Table 3). This decreased PC content was also found in isolated surfactant from animals exposed to 10 ppm NO_2 for 3 days. Sphingomyelin as a marker for an influx of serum into the alveolar airspace was also unchanged.

Surface activity studies of identical amounts of extracted lavage-phospholipids showed that lipid adsorption and equilibrium surface tension was achieved after 60 min. No difference in the equilibrium surface tension for controls and 0.8 ppm NO_2-exposed animals was found. Exposure to higher NO_2 concentrations (5 and 10 ppm) resulted in higher values for the equilibrium surface tension. The same effects were observed for the maximal and the minimal surface tension that were observed after cyclic compression of the phospholipid film at the air/saline interface (Table 4). Purified surfactant, used as a control for surface activity, had already reached its equilibrium surface tension after 15 min. For this material the minimal and the maximal surface tension values were 12.1 ± 1.1 and 43.3 ± 0.3 mN/m. When isolated surfactant from in vivo exposed animals was measured, surface tension values were higher than those for the controls. However, they were comparable to those of phospholipid extracts from lavages of exposed animals. If the isolated surfactant from controls was exposed in vitro for 4 h to a 10 ppm NO_2 atmosphere, the minimal surface tension and the maximal surface tension increased to 17.3 ± 1.9 mN/m and 50.4 ± 1.3 mN/m, respectively, indicating the direct impairment of this surfactant parameter induced by NO_2.

Table 4. Surface tension properties of rat surfactant phospholipid extracts from controls and NO_2-treated animals

Source and treatment	(n)[a]	Equilibrium (mN/m)	Maximal (mN/m)	Minimal (mN/m)
Purified surfactant				
Control	(4)	12.3 ± 0.4	43.3 ± 0.3	11.1 ± 1.1
After in vitro exposure 10 ppm/4 h	(4)	20.4 ± 0.5**	50.4 ± 1.3**	17.3 ± 1.9**
After in vivo exposure 10 ppm/3 days	(4)	19.8 ± 1.0**	50.2 ± 1.6**	19.0 ± 0.9**
Lavages				
Control	(6)	21.7 ± 0.5	50.6 ± 0.8	20.6 ± 0.7
0.8 ppm/1 day	(6)	21.7 ± 1.2	49.8 ± 1.7	21.1 ± 0.5
3 days	(6)	22.8 ± 0.5	51.2 ± 5.0	20.9 ± 0.8
5.0 ppm/1 day	(6)	23.6 ± 1.7	54.8 ± 2.4**	22.9 ± 1.8*
3 days	(6)	24.8 ± 2.0*	57.9 ± 6.2*	22.1 ± 0.1**
10.0 ppm/1 day	(6)	27.6 ± 1.1**	56.6 ± 0.8**	24.3 ± 1.7**
3 days	(6)	25.1 ± 0.4**,++	58.2 ± 3.9**	23.7 ± 1.2**

[a] n = number of experiment

Results represent mean ± SD being different from control with * = $P \leq 0.05$ and ** = $P \leq 0.01$ and being different from the corresponding exposure to 24 hour with ++ = $P \leq 0.01$.

Protein Component and SP-A

Like the phospholipids, the amount of protein exhibited a concentration and time dependence in the bronchoalveolar lavage from NO_2-exposed rats. From all proteins that are contained in this material the surfactant specific protein A, which accounts for several functions on type II pneumocytes and macrophages, was isolated and determined. Structural comparison of the SP-A in lavages from controls and NO_2-treated animals (10 ppm, 3 days) showed identity in electrophoretical mobility. Also their immunological reactivity demonstrated identity when reacting with a specific polyclonal antibody against rat SP-A. Moreover, with this antibody no SP-A degradation products were found in the immunoblots (Fig. 1).

Morphological Changes

Histological investigations of the lungs from rats exposed to 5 and 10 ppm NO_2 revealed identical alterations: thickening of the interstitium which was infiltrated by inflammatory mononuclear cells (Figs. 2 and 3). This infiltration was located mainly in the walls of the respiratory bronchioles and the corresponding alveoli. The walls of larger bronchioles exhibited no significant inflammatory infiltration, although interstitial edema, observed mainly in the perivascular spaces, was seen.

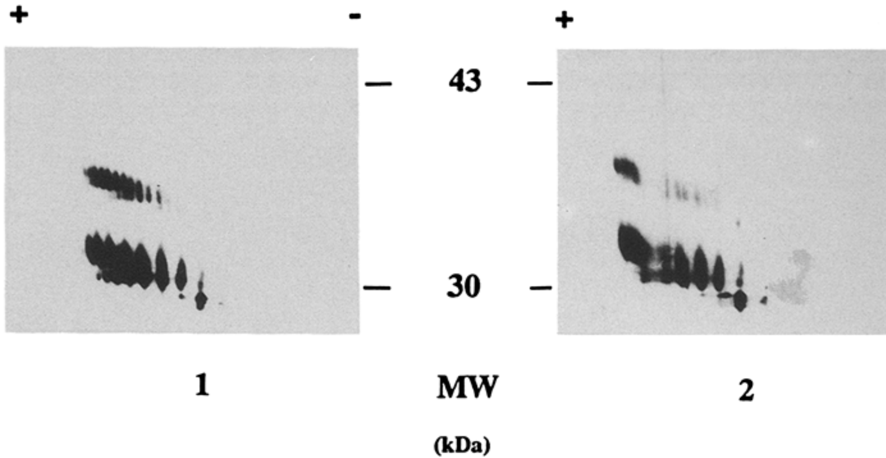

Fig. 1. Immunoblot of SP-A in lavages from rats exposed to compressed air (1) and 10 ppm NO_2 (2) for 3 days. The protein was visualized by immunostain with anti-rat SP-A serum. No degradation fragments were found after two-dimensional gel electrophoretic separation.

Discussion

Surfactant phospholipid was increased after inhalation of NO_2. This increase showed a dose dependence. In the literature there is controversy concerning the phospholipid component of the bronchoalveolar lavage material that has been exposed to NO_2 [4, 5, 25]. This may be due to different experimental designs. Influx of serum into the alveolar space, however, could not explain this increase because a higher amount of sphingomyelin from the serum was not observed in the lavage. Possibly the phospholipids of cells that are destroyed during exposure are responsible for this increase. Since the NO_2 forms free radicals and induces lipid autoxidation, we were surprised that phospholipid composition was only slightly changed after exposure. Protective effects of endogenous antioxidants probably account for these small changes [14]. Increase in oxidant concentration to 10 ppm overcame these protective mechanisms. Of all fatty acids contained in phosphatidylcholine (PC), the saturated species, especially palmitic acid (16:0), accounts for the surface activity of lung surfactant. For this reason the content of saturated and unsaturated fatty acids of PC was analyzed. Surprisingly the percentage of saturated fatty acids decreased with increasing concentrations of NO_2, whereas the content of the unsaturated fatty acids increased. These changes were due mainly to the decrease in palmitic acid after exposure to 10 ppm NO_2 for 3 days. For arachidonic acid (20:4) NO_2 caused the reverse effect (Table 4).

Unexpectedly we found an elevation in the total amount of unsaturated fatty acids of phosphatidylcholine, whereas the amount of saturated fatty acids decreased. Generally, free radicals tend to add chemically to double bonds and therefore should result in a decrease of double bond-containing fatty acids. The

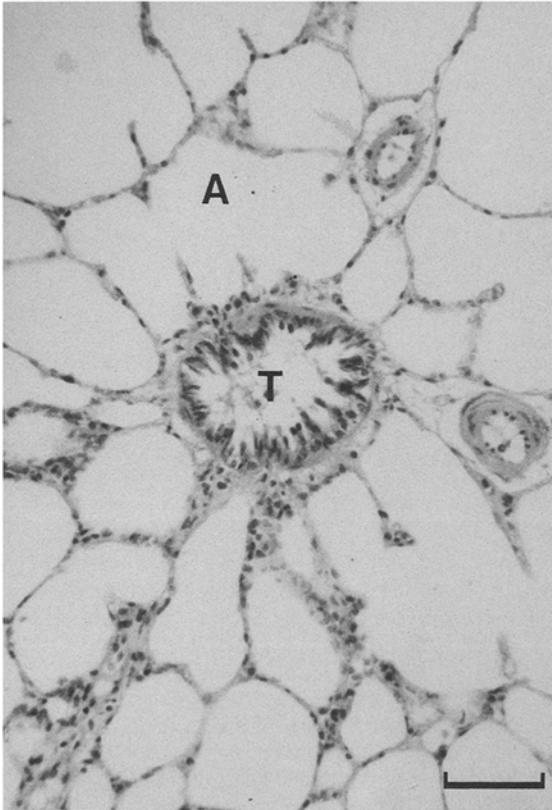

Fig. 2. Lung of a control rat showing no pathological changes. *Bar* length 45 μm (A, alveolar space; T, bronchiolus terminalis).

amount of fatty acids found in the PC was calculated from gas chromatography analyses of the main fatty acids. With this calculation only the well distributed fatty acids were recorded, thus resulting in high amounts of the disaturated forms. However, comparisons of control and experimental groups clearly showed the dependence of the decrease in saturated fatty acid on the increase of NO_2 concentration. For palmitic acid, the most prominent fatty acid, this decrease was also demonstrated. For arachidonic acid, the increase led to the hypothesis that this fatty acid is detoxified by incorporation into phosphatidylcholine. A similar finding was reported earlier [19].

The results of the surface tension studies demonstrated that phospholipid extracts from lavages could be used directly to measure this physiologic parameter. The values appear to be higher than some found in the literature that were measured with the bubble surfactometer method. The difference is believed to be based on the use of different measuring techniques. For comparative analyses, purification of surfactant seems not to be necessary to evaluate this surfactant function. However, from the surface tension studies with purified surfactant

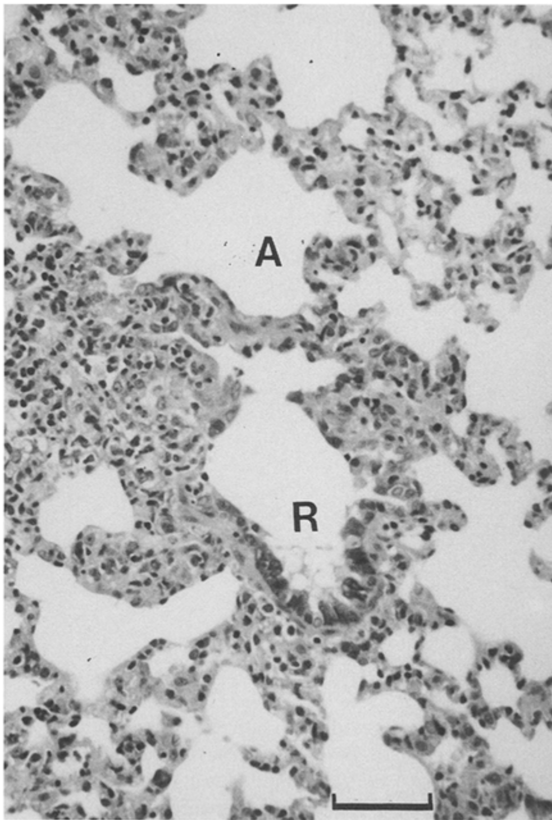

Fig. 3. Lung of an NO_2-exposed rat (10 ppm for 72 h). The walls of terminal and respiratory bronchioles, and alveolar septae show a dense interstitial mononuclear inflammatory infiltration. *Bar* length 45 μm, (A, alveolar space: R, bronchiolus respiratorius).

from controls and from NO_2-exposed animals it became clear that serum contaminants decreased minimal and maximal surface tensions. In addition to this the purified surfactant of lavages from exposed animals also revealed that the NO_2 itself impaired this surface tension lowering capacity. Moreover, it became clear that phospholipid extracts from lavages could be used directly to evaluate changes of surface tension values as a function of atmospheric NO_2 concentration. The phospholipid extracts also contain two hydrophobic surfactant specific proteins, SP-B and SP-C, that interact with the phospholipids for surface activity [12, 29]. Therefore it must be considered that the reduced activity for identical amounts of phospholipid from exposed animals may also serve as an indirect measure of SP-B and SP-C content and/or function.

The histological findings of severe inflammatory reactions in the lower respiratory tract after inhalation of nitrogen confirmed earlier studies by others [3, 10], thus demonstrating the effectiveness of the exposure procedure performed. Due to repair mechanisms in the terminal lung after NO_2 caused inflammation, and immigration of inflammatory cells, mainly granulocytes, into

the alveolar airspaces occurred. Moreover, serum proteins could have reached the alveolar air spaces via epithelial leakage, and thus increased alveolar protein pool size. Additionally, NO_2 will deteriorate cells [10], and the liberated cytosolic protein will therefore account, at least in part, for the elevated lavage protein. As demonstrated in an earlier study, the amount of the surfactant specific protein SP-A in the lavage increased with increasing concentrations of NO_2. However, several functional capacities such as aggregation with lipids, binding to immobilized mannose, and secretion inhibition of PC from isolated type II pneumocytes were impaired [9, 20]. In immunoblot analyses performed in this study it was demonstrated that neither structural alterations nor altered immunoreactivity nor tryptic degradation occurred in lavages as a result of NO_2 exposure. Nevertheless, it is possible that NO_2 affects the SP-A directly and that, via a mechanism that regulates the amount of SP-A in the lung, compensates for the toxicologic effects of the oxidative gas on this protein. However, it is still unclear if, or to what extent, the specific apoprotein A is altered in structure and function by the gas itself to explain the earlier reported impaired functions [20].

To our knowledge this is the first study that demonstrates concentration effects of in vivo NO_2 exposure on surfactant components and functions. In environmental and cigarette smoke conditions there are combination effects with other air contaminants. From the presented NO_2 exposure studies the effects of at least one prominent oxidant on surfactant components were evaluated. Lung surfactant parameters, such as surface tension or SP-A function, might serve as markers for an altered surfactant after inhalation of NO_2. However, there is also a need to analyze further the metabolic processes of the surfactant producing type II pneumocytes after exposure to oxidative stress. These in vivo studies, together with in vitro experiments, contribute to the knowledge of pathophysiological reactions in the lung caused by inhalative oxidative noxes.

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