

Nitrogen dioxide induces apoptosis and proliferation but not emphysema in rat lungs

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Key Words: apoptosis, proliferation, airspace enlargement, emphysema, stereology

Abstract

Background: Apoptosis of alveolar septal cells has been linked to emphysema formation. Nitrogen dioxide, a component of cigarette smoke, was shown to induce alveolar epithelial cell apoptosis *in vitro*. We hypothesized that nitrogen dioxide exposure of rats may result in increased alveolar septal cell apoptosis *in vivo* with ensuing emphysema, i.e. airspace enlargement and loss of alveolar walls.

Methods: Fischer 344 rats were exposed to 10 ppm nitrogen dioxide for 3, 7, 21 days or 21 days followed by 28 days at room air. Age-matched control rats were exposed to room air for 3, 21 or 49 days. Lungs fixed at 20 cm fluid column, embedded into paraffin, glycol methacrylate, and araldite were analyzed by design-based stereology. We quantified alveolar septal cell apoptosis (TUNEL assay, active caspase-3) and proliferation (Ki-67), airspace enlargement, total alveolar surface area, and absolute alveolar septal volume as well as the ultrastructural composition of the alveolar wall.

Results: Nitrogen dioxide resulted in 8-fold increase of alveolar septal cell apoptosis at day 3, and 14-fold increase in proliferation compared with age-matched controls. Airspace enlargement, indicated by 20% increase in mean airspace chord length, was evident by day 7, but was not associated with loss of alveolar walls. In contrast, nitrogen dioxide resulted in increased total surface area and absolute volume of alveolar walls comprising all compartments. The ratio of collagen to elastin, however, was reduced at day 21. Lungs exposed to nitrogen dioxide for 21 days exhibited quantitative structural characteristics seen in control lungs day 49.

Conclusions: Nitrogen dioxide exposure of rats results in increased alveolar septal cell turnover leading to accelerated lung growth, which was associated with an imbalance in the relative composition of the extracellular matrix, but fails to induce emphysema.

Introduction

Emphysema is defined as the "*abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls*" (1). The pathogenetic pathways leading to emphysema are still a matter of debate. At present, there are two major concepts, which, although not necessarily contradictory, have clearly different perspectives of the sequence of pathogenetic events. The classical concept focuses on the inflammation associated imbalance of proteases and anti-proteases. This is thought to be the primary cause of degradation of matrix components, which subsequently results in the loss of alveolar septal walls (2). On the basis of recent studies, an alternative concept has been developed, which focuses on the apoptosis of endothelial and/or alveolar epithelial cells as being the primary event in the pathogenesis of emphysema (3, 4).

Experimental induction of apoptosis of pulmonary endothelial cells as e.g., by blockade of vascular endothelial growth factor (VEGF) receptor 2 (5) or lung-targeted inactivation of VEGF (6), or of alveolar epithelial cells by transfer of active caspase-3 (7) or cigarette smoke exposure (8) were shown to result in airspace enlargement. Additional evidence for the importance of apoptosis in the pathogenesis of emphysema comes from several human studies, which demonstrated increased levels of apoptotic alveolar septal cells in emphysema patients (9-11). Notably, programmed cell death appeared to be predominant in alveolar epithelial cells of emphysema patients, whereas apoptosis of endothelial cells was less frequently observed (10, 11).

The major risk factors for chronic obstructive pulmonary disease (COPD), which comprises chronic bronchitis and emphysema, are cigarette smoking and indoor air pollution from burning fuels (12). Until now, however, studies of experimental animal models based on induction of emphysema by inhalation of e.g., cigarette smoke revealed contradictory results regarding the involvement of apoptosis of alveolar septal cells in the development of emphysema (8, 13).

Nitrogen dioxide (NO₂) is an important component of cigarette smoke with reported emissions of up to 0.73 mg NO₂ per cigarette (14). Exposure of rats to NO₂ results in airway inflammation, which, as in human COPD, is dominated by alveolar macrophages and neutrophilic granulocytes (15, 16). Further, NO₂ induces apoptosis of alveolar epithelial cells *in vitro* with actively dividing cells and cells at the leading edge of a wound being particularly susceptible (17). As short-term exposure of rats to NO₂ *in vivo* results in an initial phase of alveolar epithelial injury followed by a phase of epithelial repair (18), we hypothesized that the exposure of rats to a NO₂-containing atmosphere may result in an increase in apoptosis of alveolar epithelial cells *in vivo* with ensuing emphysema.

Materials and Methods

Experimental protocol

Forty-two male Fischer 344 rats (Charles River, Sulzfeld, Germany), 181 (5) g body weight, 8-10 weeks of age, were divided into 7 groups. Three groups (n=6 each) were exposed for 3, 7, or 21 days (23 hours/day, 7 days/week) to an atmosphere containing 10 ppm NO₂ (Messer Griesheim, Duisburg, Germany) as described earlier (19). An additional group (n=6) was exposed to NO₂ for 21 days followed by exposure to room air for 28 days. The NO₂ concentration was monitored by a

NO₂ sensitive electrochemical element (ECS 102-1, MP Sensor System, Munich, Germany). Age-matched control groups (n=4-8) were exposed to room air for 3, 21, or 49 days. Mean food consumption per day was monitored. Body weights were measured at the end of each exposure period. The animal experiments have been approved by the regional government (Regierungspräsidium Giessen, Dezernat V 54, Giessen, Germany).

Fixation and tissue sampling

Immediately after exposure, rats were sacrificed. Lungs were fixed by airway instillation with 4% phosphate-buffered paraformaldehyde at a pressure of 20 cm fluid column. Following overnight immersion into fresh fixative, lung volume was determined by fluid displacement, and two to three fractions of lung slices were collected by systematic uniform random sampling as described earlier (20).

Embedding into glycolmethacrylate (GMA) and Araldite

One fraction of lung slices and a systematic uniform random sub-sample of tissue blocks from the second fraction of slices were post-fixed in 1% glutardialdehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate buffer followed by osmication, and *en bloc* staining with aqueous uranyl acetate prior to dehydration. Complete lung slices were embedded into GMA (20) for stereological analysis of emphysema. Tissue blocks sub-sampled from the second fraction of slices of lungs exposed for 3 or 21 days to NO₂ or room air were embedded into Araldite for quantification of the components of the alveolar wall by means of transmission electron microscopy.

Embedding into paraffin

The third fraction of lung slices was dehydrated and embedded into paraffin for stereological analysis of cell proliferation and apoptosis, and identification of apoptotic cell types by means of double labeling.

Demonstration of cell proliferation and apoptosis

Cell proliferation was assessed by immuno histochemistry using the proliferation marker Ki-67 as described earlier (21). Apoptosis was assayed using terminal transferase dUTP nick end labeling (TUNEL) and immuno histochemistry for active caspase-3 (22). To identify the phenotype of apoptotic cells, double stainings by TUNEL assay and immuno histochemistry for epithelial and endothelial cell markers were performed as described earlier (22).

Stereology at the light microscopical level

Richardson stained GMA sections were used for stereological quantification of airspace enlargement (independent measurements of mean airspace chord length, alveolar surface density, and volume-weighted mean airspace volume), total alveolar surface area, and total volume of alveolar septal wall tissue according to standard techniques by point and intersection counting (20). In addition, point counting was performed on GMA sections to quantify the total volumes of inflammatory cells, alveolar macrophages, and intraalveolar polymorphonuclear granulocytes. Paraffin sections stained for Ki-67 and for DNA single strand-breaks by TUNEL assay were used for stereological quantification of cell proliferation and apoptosis, respectively (21, 22). Briefly, the fractions of alveolar wall occupied by proliferating or apoptotic alveolar septal wall cells were assessed by counting the number of test line

intersections with alveolar wall associated with cells stained for Ki-67 or TUNEL, respectively, relative to the total number of intersections with alveolar wall surface. As we were mainly interested in the effect of exposure on the alveolar septal wall architecture, only those cells were included in the analysis that were part of the alveolar septal wall, whereas free apoptotic or proliferating cells present in the alveolar air space were not considered. Analyses were performed on a computer-based Olympus BX 51 light microscope equipped with a Cast-Grid 2.01 system (Olympus, Denmark).

Stereology at the electron microscopical level

Ultrathin sections of Araldite embedded tissue blocks were collected on a 200 μm mesh grids and stained according to (23). For quantification of the volume fractions of the components of the alveolar wall, i.e. alveolar epithelium, capillary endothelium, and interstitial tissue including elastin and collagen deposition, point counting was performed on each upper left corner of the grid. A transparent sheet with 140 equidistant testpoints was superimposed onto a TV-monitor, which was connected to a Zeiss EM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany), and used for counting all points hitting the respective component relative to the total number of hits on the alveolar wall at a final magnification of 60.632 x. As described earlier (24), the total volume of each component was obtained by multiplying the volume fraction times the volume of the reference space, the alveolar wall.

Statistics

Mean values (SD) are given unless indicated otherwise. Differences between experimental groups were tested for significance with parametric One-Way Analysis of Variance (ANOVA) followed by post hoc multiple comparisons (Tukey's test) provided that normality and equal variance were given at $p > 0.1$. Otherwise, non-parametric One-Way ANOVA on ranks was used. Student's t-test was used to test for differences between pairs of groups. Significance of differences between groups was considered for $p < 0.05$. Spearman rank order correlation analysis was performed to test for relationships between proliferation, apoptosis, and inflammation. All statistical analyses were performed using the SigmaStat 3.1 software program (Jandel Scientific, Erkrath, Germany). p values < 0.05 were considered to be significant.

Results

General effects of nitrogen dioxide exposure

The initial mean body weight of the animals prior to exposure was 181 (5) g. Rats exposed to NO_2 displayed no significant gain in body weight during the first week of exposure (Table 1).

TABLE 1 BODY WEIGHTS AND FOOD CONSUMPTION

experimental group	exposure time [days]	number of animals	body weight [g] *	mean food consumption [g/day] *
all animals	0	42	181 (5)	--
room air	3	4	210 (3) ^{#¶\$‡}	15.9 (2.2) ^{§\$†‡}
room air	21	8	250 (13) ^{*¶§\$†‡}	13.6 (1.2) ^{¶§\$†‡}
room air	49	6	297 (13) ^{**§\$†}	18.6 (2.7) ^{#§\$†}
10 ppm NO ₂	3	6	189 (9) ^{#§\$†‡}	5.8 (3.6) ^{**¶†‡}
10 ppm NO ₂	7	6	183 (5) ^{*#§\$†‡}	7.0 (2.8) ^{**¶†‡}
10 ppm NO ₂	21	6	205 (11) ^{#¶\$‡}	9.4 (1.8) ^{**¶§\$†‡}
10 ppm NO ₂	21	6	201 (8) ^{#§\$†‡}	9.2 (1.9) ^{**¶§\$†‡}
/ room air	+ 28	6	280 (11) ^{**§\$†}	19.8 (2.1) ^{**§\$†}

mean values (SD)

Significant differences among groups at $p < 0.05$ (one-way ANOVA followed by posthoc pairwise multiple comparisons procedures, Tukey's test) are indicated as follows:

* versus room air 3d; # versus room air 21d; ¶ versus room air 49d;

§ versus NO₂ 3d; \$ versus NO₂ 7d; † versus NO₂ 21d; ‡ versus NO₂ / room air

During the following 2 weeks they displayed weight gain of about 20 g, whereas control rats exposed to room air for the same period of time displayed an increase in body weight of about 70 g. This was due to a significantly lower amount of mean food consumption in rats exposed to NO₂, which was 36% of the mean food consumption of control animals during the first 3 days of exposure, and about 70% during the last 2 weeks (Table 1). Animals kept in room air for 28 days after 21 days of exposure to NO₂ consumed significantly more food than during exposure to NO₂, and achieved a body weight of 280 (11) g at the end of the experiment, which was not significantly different from the mean body weight of 297 (13) g of the control rats exposed to room air for 49 days (see Table 1).

Apoptosis and proliferation of alveolar septal cells

In rat lungs exposed to NO₂ for 3 days, numerous apoptotic cells were observed in alveolar septal walls and in the alveolar space by TUNEL assay (Figure 1a) and immuno histochemistry for active caspase-3 (Figure 1b). In contrast, apoptotic cells were extremely rare in lungs of animals exposed to room air (not shown). At day 3 of exposure to NO₂, the fraction of alveolar surface area that was associated with apoptotic cells of the alveolar septal wall was significantly increased to more than 8-fold in comparison with age-matched control animals (Figure 1e). This increase exclusively reflects the increase in apoptosis of alveolar septal wall cells, as free apoptotic cells present in the alveolar air space were not considered in our analysis. With increasing exposure time, a continuous decrease of apoptotic events in alveolar septal wall cells was recorded. However, the level of alveolar septal wall apoptosis remained significantly higher than in control animals exposed to room air.

Double stainings using the epithelial and microvascular endothelial markers surfactant protein D (SpD) and aquaporin-1, revealed that by far most of the

apoptotic cells were of epithelial origin (Figure 1c). Apoptosis of microvascular endothelial cells was not observed (Figure 1d).

Immuno histochemistry for the proliferation marker Ki-67 (Figure 2a-d) revealed large numbers of proliferating cells at day 3 of exposure to NO₂. The fraction of alveolar surface area that was associated with proliferating alveolar septal cells was significantly increased to approximately 14-fold above the level of the respective age-matched control group (Figure 2e). At day 7, the amount of proliferating cells had dramatically decreased and was no longer significant from control group levels.

Correlation analysis (performed within NO₂-exposed lungs only) revealed a significant positive correlation ($r=0.604$, $p=0.008$) between apoptosis and proliferation of structure forming alveolar septal wall cells, whereas no relationship was seen with any parameter characterizing the inflammatory cells.

Inflammation

Rat lungs exposed to NO₂ for 3 days exhibited a 3-fold increase in the total volume of alveolar macrophages compared with lungs exposed to room air (Figure 3a), whereas polymorphonuclear granulocytes were only slightly but insignificantly increased (Figure 3b). After 21 days of exposure to NO₂, the total volumes of alveolar macrophages and of other inflammatory cells were increased by about 2-fold (Figure 3a,c).

Airspace enlargement

Control lungs exposed to room air displayed a progressive increase in airspace size with age (Figure 4a-e). In comparison with age-matched control lungs, animals exposed to NO₂ for 3 days demonstrated normal indices of airspace size, i.e. mean airspace chord length, alveolar surface density, and volume-weighted mean airspace volume, respectively (Table 2). However, airspace enlargement was evident at day 7 of exposure to NO₂ as was indicated by significant increases in mean airspace chord length, surface density and volume-weighted mean airspace volume. Airspace enlargement further progressed until day 21: lungs exposed to NO₂ displayed an increase in mean airspace chord length of about 20% and in volume-weighted mean airspace volume of about 40% in comparison with age-matched control lungs. Although mean chord length significantly decreased in lungs exposed to NO₂ for 21 days followed by 28 days exposure to room air in comparison with lungs exposed to NO₂ for 21 days, volume-weighted mean airspace volume and surface density were not significantly different between these two groups (Table 2). Hence, airspace enlargement appeared to persist after termination of exposure to NO₂. Equivalent indices of airspace size were noted for age-matched animals exposed to room air for 49 days. The major difference between lungs exposed to NO₂ versus room air was that airspace size of lungs exposed to NO₂ had reached values, which were equivalent to 49 days control lungs already by day seven.

Loss of alveolar walls

Despite the fact that airspace enlargement was prominent in lungs exposed to NO₂ for 21 days as compared with age-matched control lungs, we did not find any sign for a loss of alveolar walls. In contrast, absolute volume and total surface area of alveolar septal walls were increased in NO₂-exposed rats (day 21) by 53% and 29%, respectively, compared with age-matched controls (Table 2, Figures 5, 6). Alveolar

septal wall volume continued to increase after proliferation and apoptosis had returned to normal. There were no significant differences in the absolute volume of the non-parenchymal compartment. The increases in volume and surface area of alveolar septal walls were even more prominent when mass-specific parameters are considered (data not shown). The quantitative ultrastructural analysis revealed that the increased alveolar septal wall volume at day 3 of NO₂-exposure was exclusively due to an increase in the volume of the alveolar epithelium (Figure 7). Whereas no further increase was noted in the alveolar epithelial volume of NO₂-exposed lungs, the volumes of capillary endothelium and interstitial tissue as well as of collagen and elastin were increased at day 21 compared with age-matched control lungs. Notably, the collagen-to-elastin ratio was significantly lower in NO₂-exposed lungs at day 21 compared to control lungs exposed to room air for 21 days (Figure 8).

A progressive age-dependent increase was noted in control lungs in all structural parameters related to the gas exchange region. Notably, total alveolar surface area and absolute alveolar septal volume of NO₂-exposed rats (day 21) were largely equivalent to values characteristic of control animals at day 49. There was no significant alteration in alveolar septal wall volume and total alveolar surface area after exposure to NO₂ had been terminated. and no changes were noted after an additional exposure to room air for 28 days.

TABLE 2 QUANTITATIVE MORPHOLOGICAL DATA OBTAINED BY LIGHT MICROSCOPY

Absolute Volumes [mm ³]	room air			10 ppm NO ₂			NO ₂ / room air
	3d	21d	49d	3d	7d	21d	21d + 28d
Fixed Lung	4920 (549)	5496 (435)	7140 (213)*	4768 (592)	6322 (661) [#]	7623 (514) [§]	7665 (1107) [§]
Non-Parenchyma	689 (76)	703 (104)	725 (86)	626 (108)	758 (177)	733 (62)	752 (129)
Parenchyma	4231 (547)	4793 (352)	6415 (190)*	4142 (564)	5564 (553) [#]	6891 (501) [§]	6913 (1002) [§]
Airspace	3768 (525)	4316 (332)	5765 (204) [§]	3546 (556)	4941 (486) [§]	6161 (487) [§]	6275 (962) [§]
Alveolar Macrophages	47 (22)	47 (20)	39 (12)	155 (59) [∞]	75 (29)	103 (43) [¶]	34 (6)
Granulocytes (PMNs)	5.6 (2.8)	7.1 (3.2)	7.4 (3.3)	13.6 (7.8)	9.9 (5.5)	16.0 (8.9)	7.8 (0.3)
Other Cells	8.2 (3.6)	14.0 (6.8)	15.4 (5.1)	19.2 (4.4)	16.8 (7.4)	27.3 (11.0) [∞]	13.0 (2.3)
Alveolar Septal Wall	463 (62)	477 (40)	650 (52) [§]	596 (86)	624 (112) [§]	729 (93) [§]	638 (74) [§]
Mean Thickness [μm]	4.2 (0.5)	4.0 (0.2)	4.1 (0.4)	5.3 (0.6) [∞]	5.0 (0.5) [∞]	4.7 (0.4)	4.2 (0.3)
Mean Chord Length [μm]	56.1 (5.7)	65.5 (4.4)	73.3 (10.2)*	56.2 (6.7)	76.6 (7.6)*	78.9 (8.0)*	72.8 (9.0)*
Volume-Weighted Mean Airspace Volume [10 ⁶ μm ³]	4.83 (1.35)	5.36 (1.92)	6.36 (1.92) [§]	3.17 (0.45)	6.88 (2.11) [§]	7.49 (0.88) [§]	7.15 (0.73) [§]
Surface Density [1/mm]	52.4 (3.7)	49.7 (5.3)	48.7 (2.7)	54.9 (6.1)	44.4 (3.3)*	44.8 (4.1)*	45.3 (2.3)*
Total Alveolar Surface Area [cm ²]	2205 (214)	2377 (240)	3125 (161) [§]	2253 (180)	2479 (384)	3078 (269) [§]	3121 (433) [§]

Significant differences among groups at $p < .05$ (one-way ANOVA followed by posthoc pairwise multiple comparisons, Tukey's test) are indicated as follows: [∞] versus room air 3d, 21d, 49d; NO₂ / room air; [#] versus room air 3d, 21d; NO₂ 3d, 21d; NO₂ / room air; * versus room air 3d, 21d; NO₂ 3d; [§] versus room air 3d, 21d; NO₂ 3d, 7d; [§] versus room air 3d, 21d; * versus room air 3d; NO₂ 3d, 21d; * versus NO₂ 3d; [¶] versus NO₂ / room air

Discussion

The effects of exposure of experimental animals to NO₂ vary widely as e.g., with the species and strain exposed, the concentration and duration applied as well as age and gender of the animals as comprehensively reviewed by others (25). In our study of juvenile male Fischer 344 rats, which were 8-10 weeks of age at the beginning of the experiments, lungs exposed to an atmosphere containing 10 ppm NO₂ for 23 hours per day for 3 days displayed an increased turnover of alveolar septal cells indicated by an 8-fold increase in apoptotic events and a 14-fold increase in proliferation in comparison with age-matched control lungs. Lungs exposed to NO₂ for 21 days exhibited quantitative structural characteristics equivalent to control lungs, which were 28 days older. As transfer of lungs to room air after 21 days of exposure to NO₂ did not result in a reversion of this process, we suggest that NO₂ induced an accelerated growth of the lung, which, however, was associated with an imbalance in the relative composition of the extracellular matrix indicated by a decreased collagen-to-elastin ratio.

In agreement with results from *in vitro* studies (17), we observed a significant increase in alveolar septal wall cell death in lungs exposed to NO₂ *in vivo*. Double stainings for apoptosis and markers of alveolar cell types revealed that alveolar epithelial type II cells were the main target of NO₂-induced apoptotic cell death, whereas vascular and capillary endothelial cells were not affected. Although experimental induction of epithelial and/or endothelial cell apoptosis has been linked to the development of airspace enlargement in various murine models (5-7) including cigarette smoke exposure (8), apoptosis of alveolar epithelial cells appeared to predominate in human emphysema (10, 11). Recent studies demonstrated that in lungs of patients with emphysema, however, proliferation of alveolar septal cell was increased, too (9, 10). These findings indicate that emphysema may not simply be the consequence of the induction of apoptosis, but more likely appears to be the result of an imbalance between apoptosis and proliferation in humans (9, 10).

Exposure of rats to NO₂ was demonstrated to induce airway inflammation, which, as in human COPD, was dominated by alveolar macrophages and neutrophilic granulocytes (15, 16). Only minor contribution of polymorphonuclear granulocytes was seen in our experiments. Whereas some studies reported that exposure to NO₂ may finally result in emphysema (16), others did not find evidence for that (26). Unfortunately, all these studies relied on the mere measurement of airspace enlargement to assess the presence of emphysema. Demonstration of an increase in airspace size alone, however, is insufficient to conclude that emphysema had developed (27). Stereological approaches to quantify emphysema have shown that in human emphysema (28) as well as in elastase-induced emphysema in the mouse (29), airspace enlargement is accompanied by a loss of alveolar walls as evidenced by a decrease in total alveolar surface area and total alveolar wall volume. Using a stereological approach to quantify airspace enlargement by independent measurements of mean airspace chord length, alveolar surface density (S/V-ratio), and volume-weighted mean airspace volume, respectively, as well as quantification of total alveolar surface area and absolute alveolar septal wall volume, we demonstrated that increased airspace enlargement during exposure to NO₂ was not associated with a loss of alveolar walls. On the contrary, lungs exposed to NO₂ for 21 days were characterized by a 29% increase in total alveolar surface area and a 53% increase in absolute septal volume in comparison with age-matched control

lungs. Hence, relying on indicators of airspace enlargement alone would have led to the false conclusion of NO₂ inducing apoptosis with subsequent emphysema. In contrast, inclusion of indicators of loss of alveolar walls as e.g., total alveolar wall volume and total alveolar surface area revealed that the increase in apoptotic events observed in lungs exposed to NO₂ was over-compensated by the parallel increase in proliferation.

In early studies, Evans and co-workers demonstrated that NO₂ results in hyperplasia of alveolar epithelial type II cells as a response to the initial damage of type I cells (30). In line with this, our quantitative ultrastructural analysis revealed that at day 3 of exposure to NO₂ the alveolar epithelium only exhibited an increase in the total volume, whereas all other compartments of the alveolar septal wall were unaffected. Hyperplasia of alveolar epithelial type II was followed by transformation of type II into type I cells (18). While transformation is one mechanism to resolve hyperplasia, apoptosis of type II cells, as demonstrated in this study, is another mechanism, which is probably more important in quantitative terms than transformation (31). The significant correlation between the indices of proliferating and apoptotic cells in our study highlights the close link between apoptosis and proliferation of alveolar septal wall cells in this model. The decrease in both proliferation and apoptosis with exposure time may reflect some adaptive mechanisms induced e.g., in alveolar epithelial type II cells, which were previously shown to exhibit restored antioxidant superoxide dismutase activity at day 20 (32), and/or in bronchoalveolar lavage cells, which showed an increase in glutathione peroxidase and superoxide dismutase enzyme activities in this model (33). However, we cannot discriminate whether the induction of these protective mechanisms were direct effects due to exposure or indirect effect due to the reduced food intake (see below).

After 21 days of exposure to NO₂, all compartments of the alveolar septum exhibited an increase in their total volume in comparison with room air exposure. However, no equivalent increase was seen in the collagen-to-elastin ratio indicating that accelerated lung growth was associated with an imbalance in extracellular matrix deposition.

In this study, as in most studies performed in the field of emphysema research (6-8), animals were 8-10 weeks of age at the beginning of exposure, i.e. they were juvenile not adult individuals. Considerable normal lung growth is observed in rats and mice up to an age of at least 5 months, and comprises an increase in total alveolar surface area and absolute alveolar wall volume as well as an age-dependent increase in airspace size (34, 35). The quantitative morphological data obtained in our study of control animals compare well with data published on ageing male Fischer 344 summarized recently (35). In this review, the authors pointed out that only little changes occur in tissue volumes from 5 to 26 months of age with the exception of a reduction in the extracellular matrix component of the interstitium, whereas total airspace volume and airspace size increased with age (35). These structural changes are accompanied by age-related alterations in the biochemical composition of the extracellular matrix as well as in lung function (36). Age-related changes in airspace size are also well documented in humans (37), as are age-related changes in lung function (38). Both age-dependent decline in lung function and increase in airspace size are significantly accelerated in smokers (39, 40). Notably, data from a recent prospective study of California schoolchildren suggest that ambient NO₂ is a major contributor to the adverse effects of air pollution on lung development from the age of 10 to 18 years, which lead to clinically significant deficits e.g., in attained FEV₁

as children reached adulthood (41). Although in the present study, lung function parameters were not analyzed, we have recently shown that exposure to NO₂ resulted in a progressive decline in expiratory airflow associated with an increase in airspace size as well as in volume-weighted mean alveolar volume in juvenile mice (42).

From our study, we can not discriminate if the alterations observed were a direct effect of the exposure to NO₂ or an indirect effect due to the significantly reduced food consumption (day 3: 36%, day 7: 44% of control animals at day 3; day 21: 69% of control animals at day 21). Calorie restriction (two-thirds reduction for 2 weeks) in mice has recently been demonstrated to result in a reduction in body weight of about 40% with a concomitant increase in apoptosis, a 25% reduction in total alveolar surface area, and a reduction of total alveolar wall volume of about one third (43). Unlike these findings, exposure of rat lungs to NO₂ did not result in loss of body weight, but only in a reduction of weight gain, which may be due to the reduced activity, and hence metabolism, of NO₂-exposed animals. We did not observe a loss of alveolar walls as a direct effect of exposure or as an indirect effect due to reduced food intake. Instead, a 29% increase in total alveolar surface area and a 53% increase in total alveolar wall volume were observed after 21 days of exposure to NO₂ versus room air. Wright and Churg pointed out that all of the animal models of emphysema using cigarette smoke exposure that have evaluated changes in body weight have demonstrated a failure of smoke-exposed animals to gain weight (44). In a recent pair-fed study in mice, cigarette smoking for 4 weeks was shown to result in reduced weight gain equivalent to a food restriction group. In contrast to food restriction, smoke exposure caused a reduction in hypothalamic neuropeptide Y and fat mass, and regulated adipose cytokines finally resulting in the reduced weight gain (45). Unfortunately, data regarding inflammation or histopathology were not recorded in this study. From human studies it is well documented that in contrast to chronic starvation well-nourished adults who, for experimental or other reasons, have lowered their food intakes an apparently enhanced metabolic efficiency is observed resulting from changes in metabolic rates, which are disproportionate to the changes in body weight (46).

In conclusion, nitrogen dioxide exposure of juvenile rats resulted in increased alveolar septal cell turnover as evidenced by an increase in apoptosis and proliferation of alveolar septal cells. Comparison with age-matched control lungs indicates that increased cell turnover was associated with an acceleration of normal lung growth as evidenced by an increase in airspace size, total alveolar surface area as well as total alveolar wall volume. Despite the increase in both collagen and elastin, the reduced collagen-to-elastin ratio in NO₂-exposed animals indicates that extracellular matrix deposition was impaired and may result in altered lung mechanics in this model. We suggest that the alterations observed are compatible with an accelerated lung growth of rat lungs exposed to NO₂, which, however, does not comprise a proportional growth of collagen and elastin. Exposure of juvenile rats to NO₂ may be a useful model to study mechanisms leading to adverse effects of air pollution on postnatal lung development observed in human juveniles (41).

Acknowledgements

We gratefully acknowledge the help of Bernd Müller and Petra Staats with the nitrogen dioxide exposure model, and the expert technical assistance of Roswitha

Naumann and Tanja Rausch in all steps of organ preparation, tissue processing, sectioning and staining procedures.

Competing interests

none declared

Funding

The study was supported by grant of the German Ministerium für Bildung und Forschung (FKZ 01GC0103).

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Figure legends

Figure 1 Apoptosis of alveolar septal cells. Apoptotic alveolar septal wall cells (arrowheads) are demonstrated (**A**) by TUNEL assay and (**B**) by indirect immuno histochemistry against active caspase-3 in lungs exposed to NO₂ for 3 days; arrows indicate alveolar macrophages with engulfed apoptotic bodies. (**C**) Apoptotic cells are identified as alveolar epithelial type II cells by double staining using TUNEL assay (brown staining) and indirect immuno histochemistry against surfactant protein D (greenish staining), whereas (**D**) co-staining against aquaporin 1 (greenish staining) reveals that endothelial cells do not undergo apoptosis (brown staining); lungs were exposed to NO₂ for 3 days. (**E**) Quantification of TUNEL assay (as shown in A) reveals a significant increase in apoptosis of structure forming alveolar septal wall cells at day 3 and day 7 of exposure to NO₂. (A) and (B) as well as (C) and (D) were taken at identical magnifications, respectively. Significant differences between groups ($p < 0.05$, one-way analysis of variance followed by posthoc multiple comparisons according to Tukey's test) are indicated as follows: * *versus* all groups exposed to room air and *versus* NO₂ / room air; # *versus* NO₂ 21d.

Figure 2 Proliferation of alveolar septal cells. Proliferating alveolar septal wall cells (arrowheads) are demonstrated by indirect immuno histochemistry against proliferation marker Ki-67 in lungs exposed (**A**) to NO₂ for 3 days, (**B**) to room air for 3 days, (**C**) to NO₂ for 7 days, and (**D**) to NO₂ for 21 days. (**E**) Quantification of Ki-67 staining (as shown in A-D) reveals a significant increase in proliferation of structure forming alveolar septal wall cells at day 3 of exposure to NO₂. Insert in (**A**) demonstrates that the majority of Ki-67 positive cells are alveolar epithelial cells (AE); asterisk denotes inflammatory cells in the alveolar airspace. (A) and (B) as well as (C) and (D) were taken at identical magnifications, respectively. Significant

differences between groups ($p < 0.05$, one-way analysis of variance followed by posthoc multiple comparisons according to Tukey's test) are indicated as follows: * *versus* all groups.

Figure 3 Quantification of total volumes of inflammatory cells (performed on 1 μm thick glycol methacrylate sections) reveals (A) an increase in alveolar macrophages in lungs exposed to NO_2 for 3 and 21 days, (B) a slight but insignificant increase in polymorphonuclear granulocytes (PMNs) after 21 days of exposure to NO_2 , and (C) a significant increase in other inflammatory cells at day 21 of exposure to NO_2 . Significant differences between groups ($p < 0.05$, one-way analysis of variance followed by posthoc multiple comparisons according to Tukey's test) are indicated as follows: * *versus* all groups exposed to room air and *versus* NO_2 / room air; § *versus* NO_2 / room air.

Figure 4 Histopathology of terminal bronchioles and gas exchange area. H&E stainings of paraffin sections show (A) normal histology in lungs exposed to room air for 21 days, whereas lungs exposed to NO_2 for (B) 3 days, (C) 7 days, and (D) 21 days demonstrate inflammation and airspace enlargement. (E) Quantification of mean chord length as an indicator of changes in airspace size (performed on 1 μm thick glycol methacrylate sections) reveals a continuous increase in mean chord length in control lungs and an accelerated increase in lungs exposed to NO_2 . All micrographs were taken at identical magnification. Significant differences between groups ($p < 0.05$, one-way analysis of variance followed by posthoc multiple comparisons according to Tukey's test) are indicated as follows: * *versus* room air 3d and NO_2 3d; # *versus* room air 21d.

Figure 5 Quantification of total alveolar surface area as an indicator of loss of alveolar walls (performed on 1 μm thick glycol methacrylate sections) reveals a continuous increase in total alveolar wall surface in control lungs and an accelerated increase in lungs exposed to NO_2 . Significant differences between groups ($p < 0.05$, one-way analysis of variance followed by posthoc multiple comparisons according to Tukey's test) are indicated as follows: * *versus* room air 3d, 21d and *versus* NO_2 3d, 7d.

Figure 6 Quantification of total alveolar septal volume as an indicator of a potential loss of alveolar walls (performed on 1 μm thick glycol methacrylate sections) reveals an increase in alveolar wall volume in control lungs and an accelerated increase in lungs exposed to NO_2 . Significant differences between groups ($p < 0.05$, one-way analysis of variance followed by posthoc multiple comparisons according to Tukey's test) are indicated as follows: * *versus* room air 3d, 21d.

Figure 7 Ultrastructure of alveolar septal wall of (A) lung exposed to room air for 3 days. Electron microscopic quantification of different components of the alveolar septal wall revealed an increase in the total volume of (B) the alveolar epithelium already on day 3 of exposure to NO_2 whereas the other components of the alveolar septal wall were not affected. At day 21 of exposure to NO_2 , an equivalent increase in volume was seen in all components of the alveolar wall, i.e. in the (A) alveolar epithelium, (C) interstitial tissue, (D) capillary endothelium, (E) collagen fibres, and (F) elastin. Significant differences between groups ($p < 0.05$, one-way analysis of variance followed by posthoc multiple comparisons according to Tukey's test) are indicated. AE1: alveolar epithelial type 1 cell; Air: alveolar airspace; CE: capillary endothelium; Int: interstitial tissue; PMN: polymorphonuclear granulocyte.

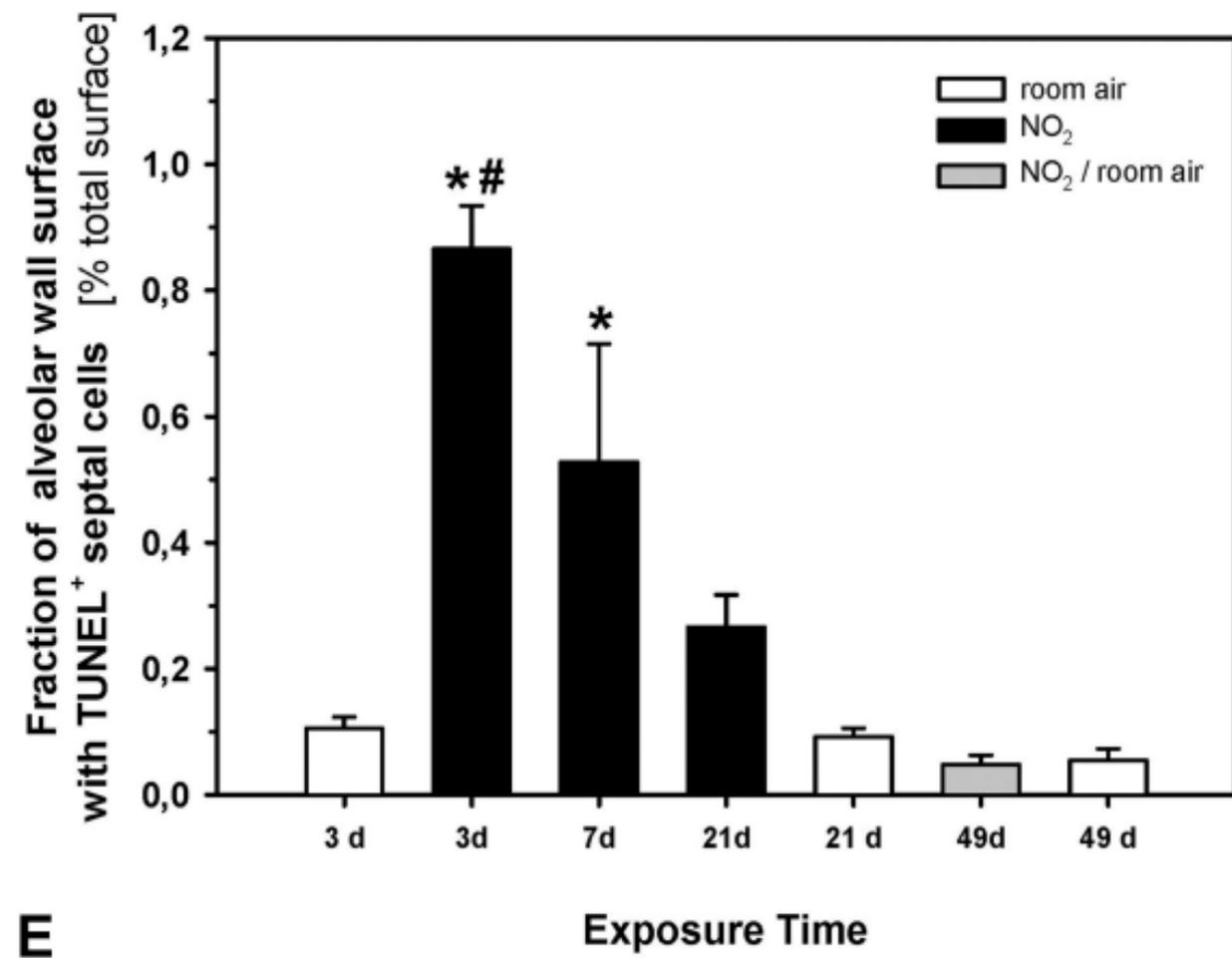
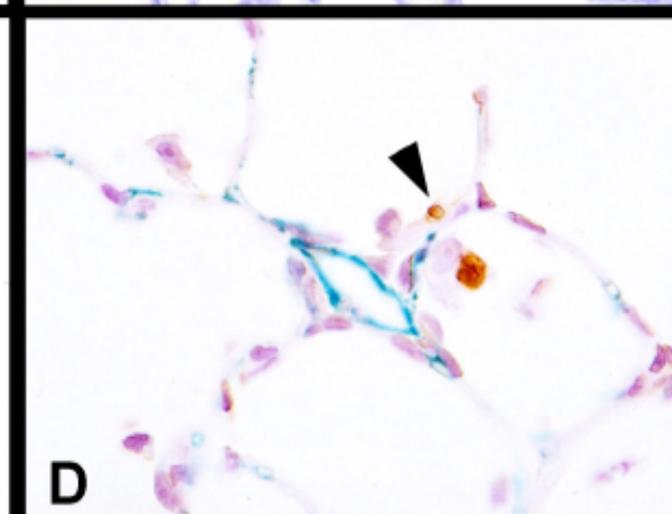
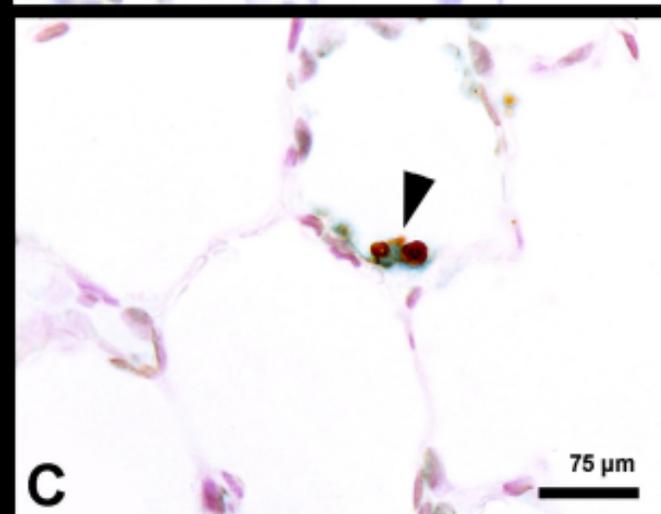
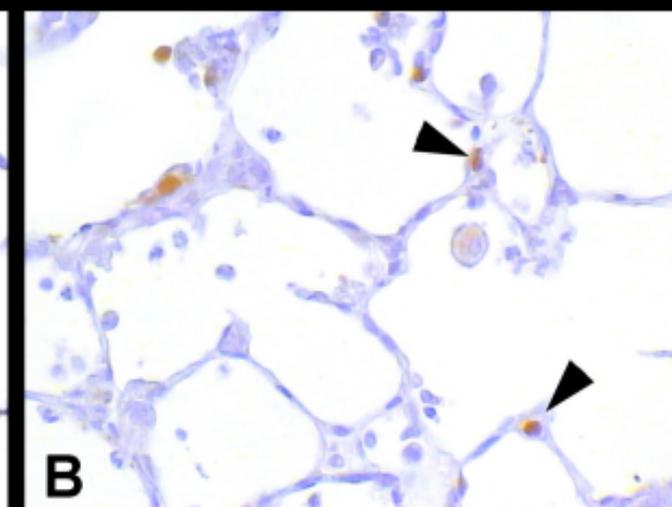
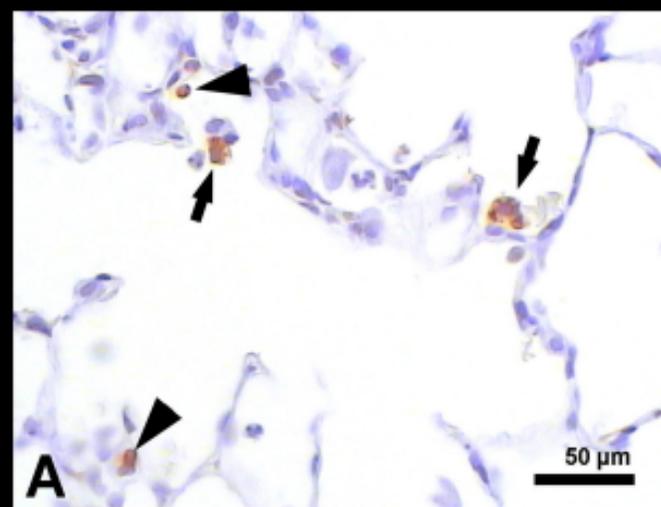
Figure 8 Ultrastructure of extracellular matrix composition of alveolar septal wall entrance ring of (A) lung exposed to room air for 3 days. Electron microscopic quantification revealed (B) a significantly decreased collagen-to-elastin ratio in lungs exposed to NO₂ for 21 days in comparison to control lungs exposed to room air for 21 day. At day 3, no differences were seen between lungs exposed to NO₂ or room air. Significant differences between groups ($p < 0.05$, Student's t-test) are indicated. AE1: alveolar epithelial type 1 cell; AE2: alveolar epithelial type 2 cell; CE: capillary endothelium; Col: collagen fibrils; E, Elast: elastin fibres; Fibr: fibrocytes.

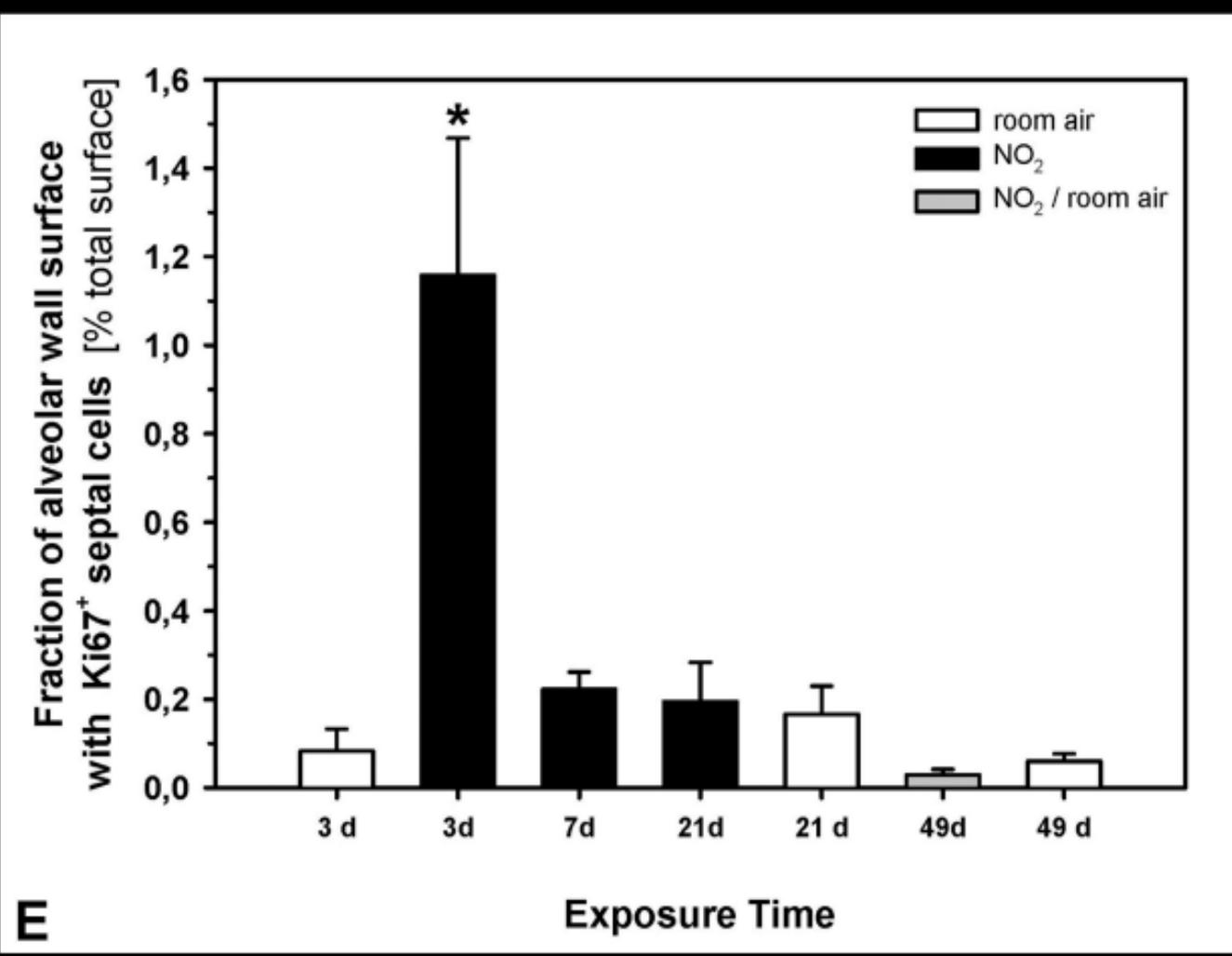
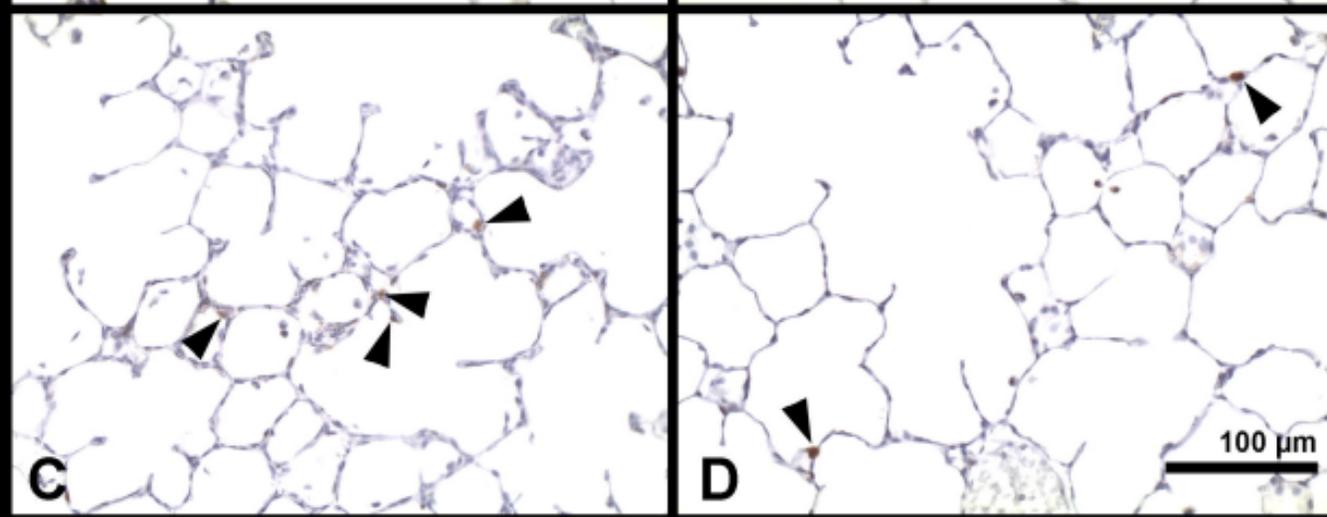
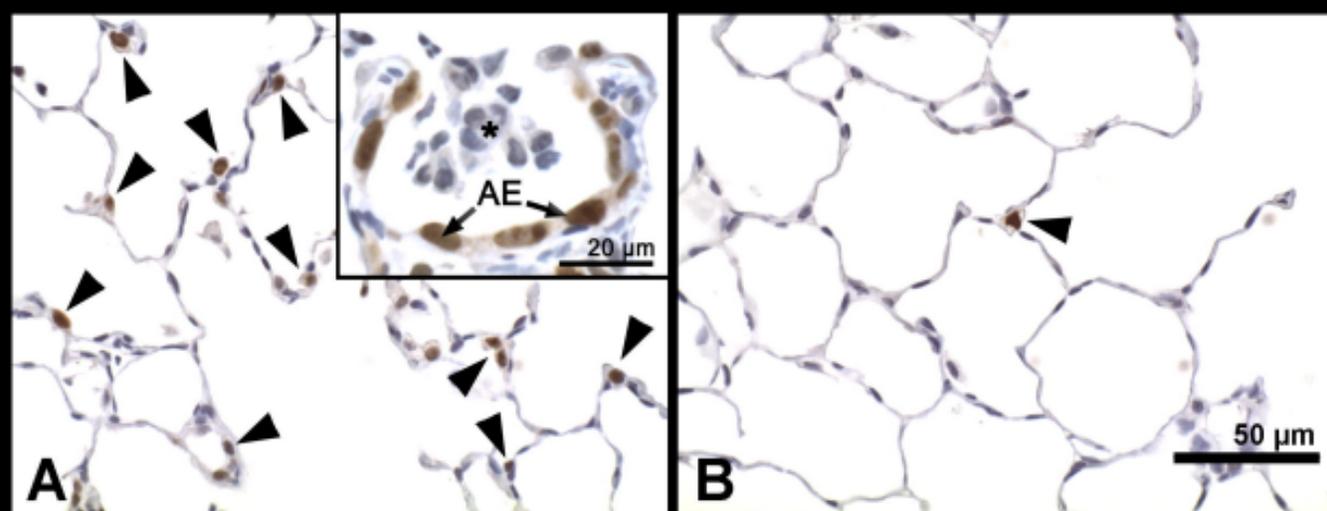
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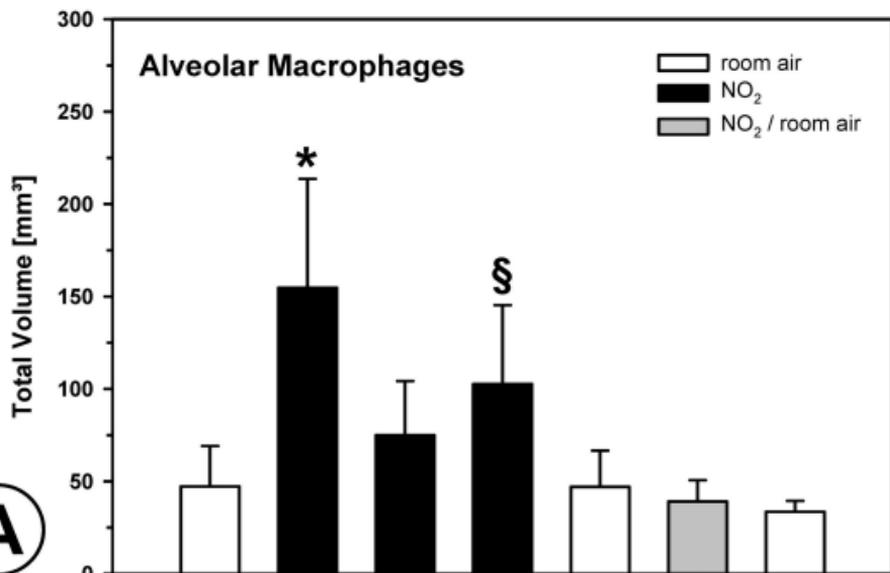




Alveolar Macrophages

room air
NO₂
NO₂ / room air

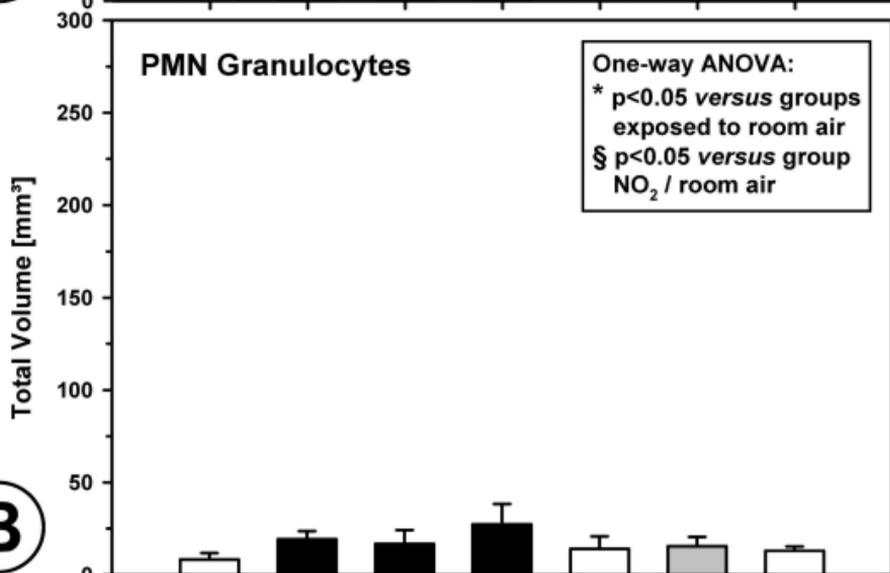
A



PMN Granulocytes

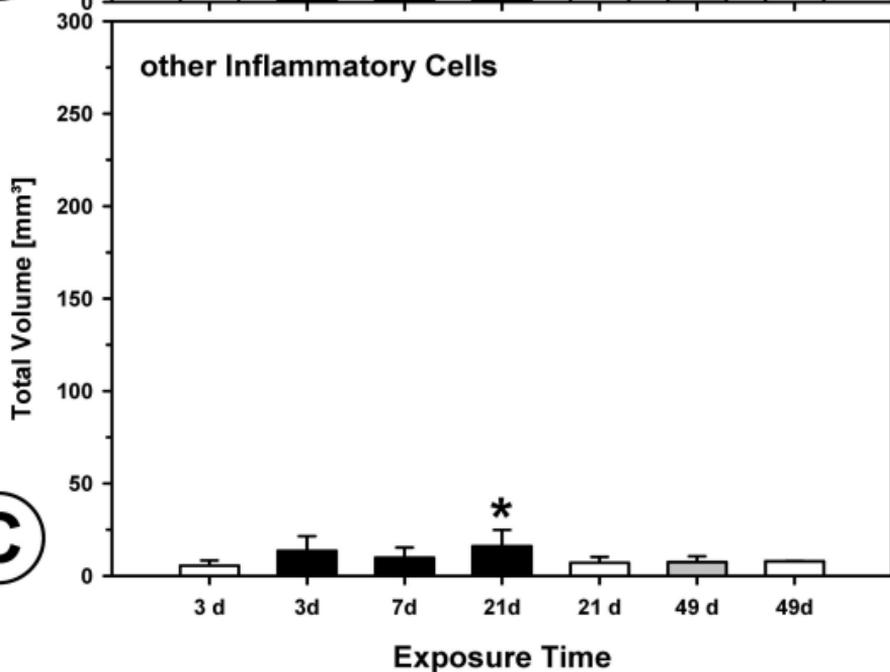
One-way ANOVA:
* p<0.05 versus groups exposed to room air
§ p<0.05 versus group NO₂ / room air

B

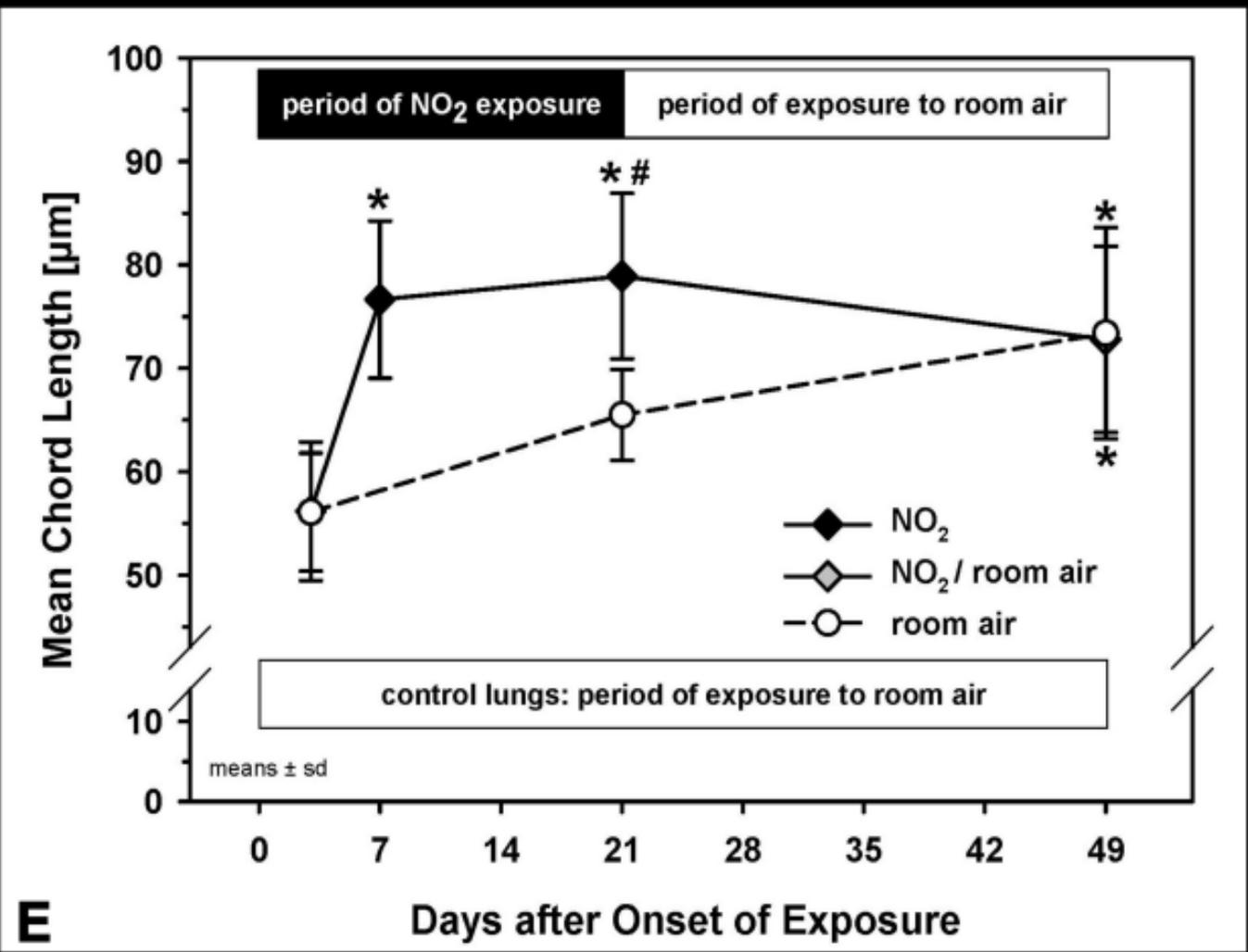
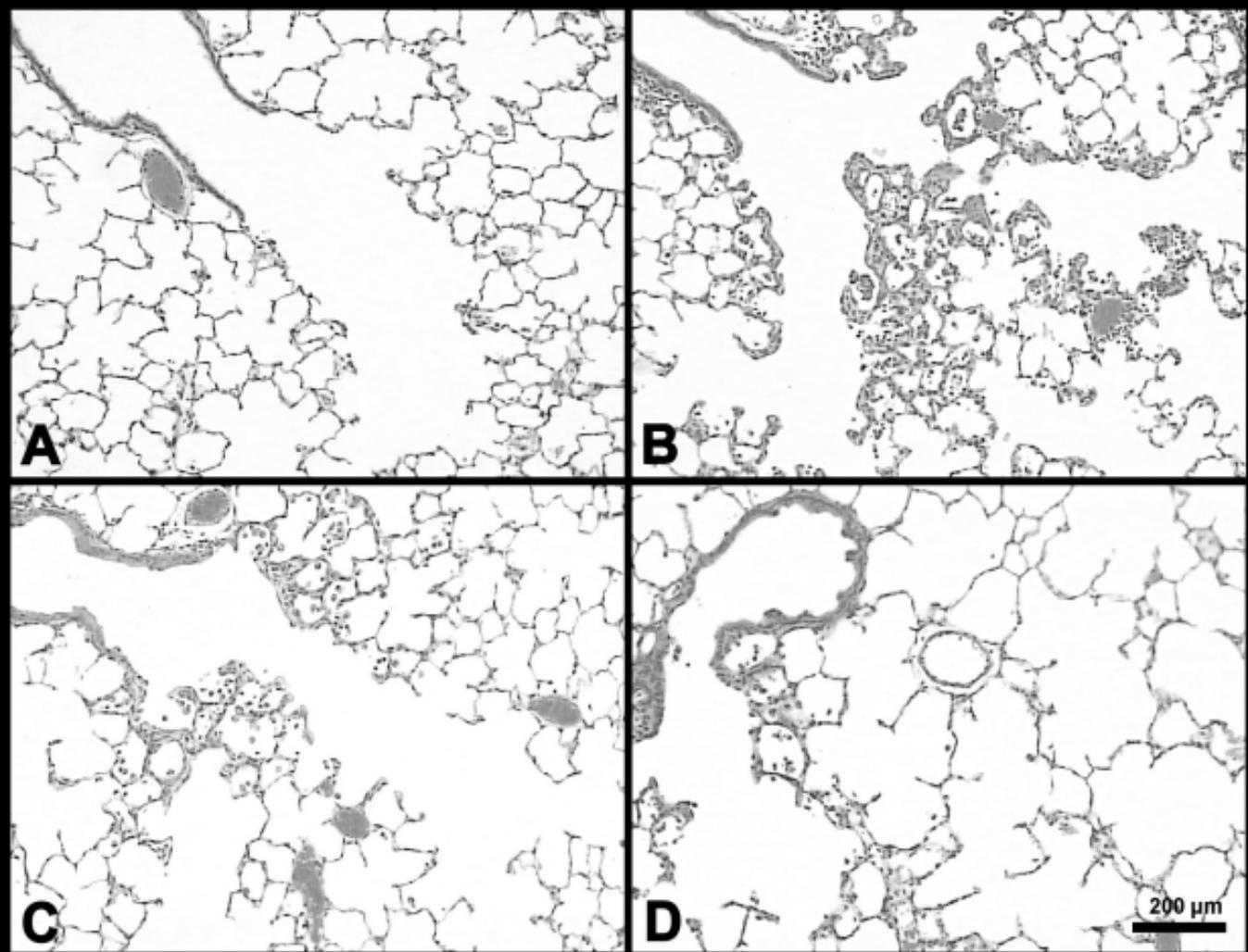


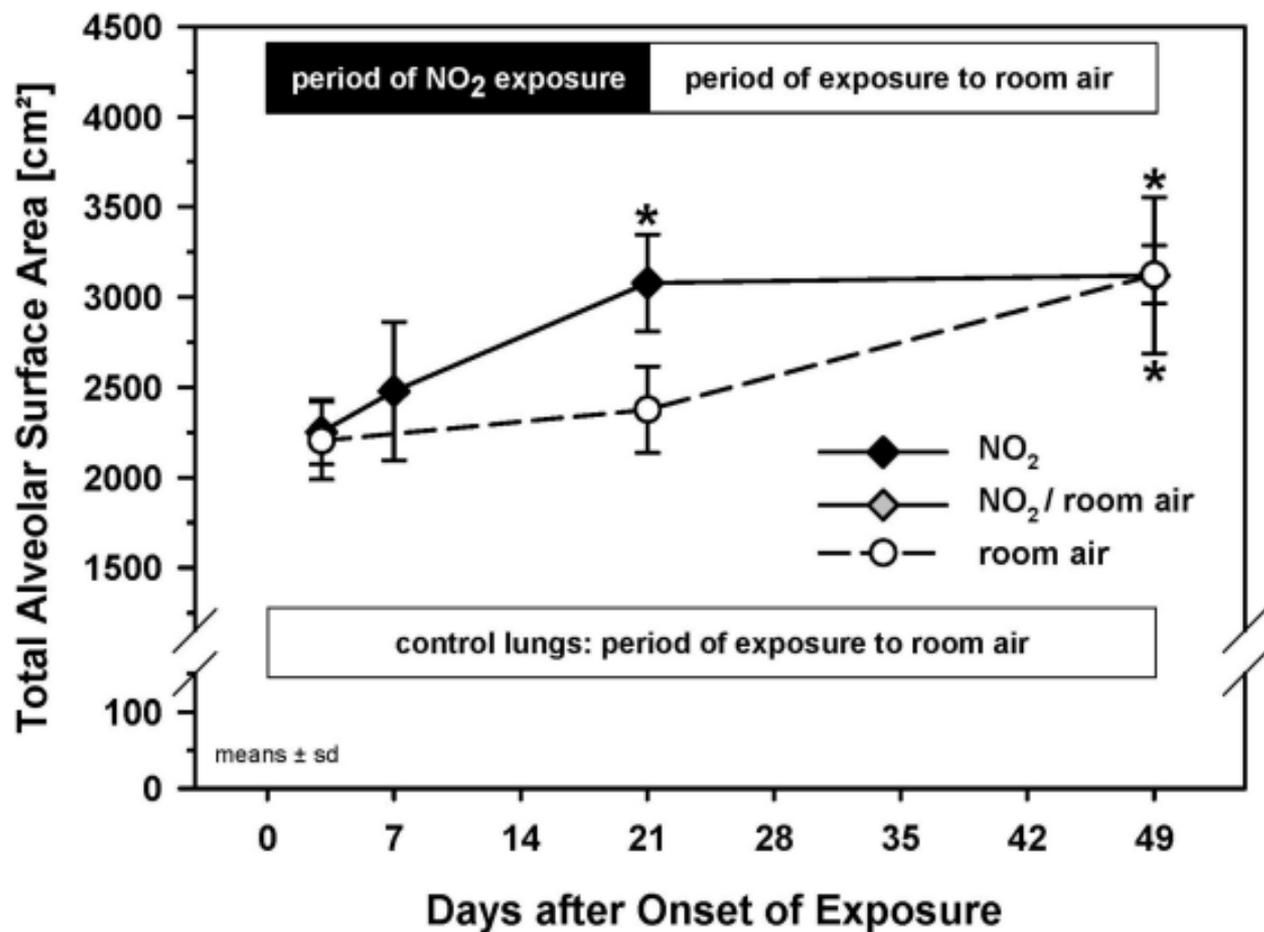
other Inflammatory Cells

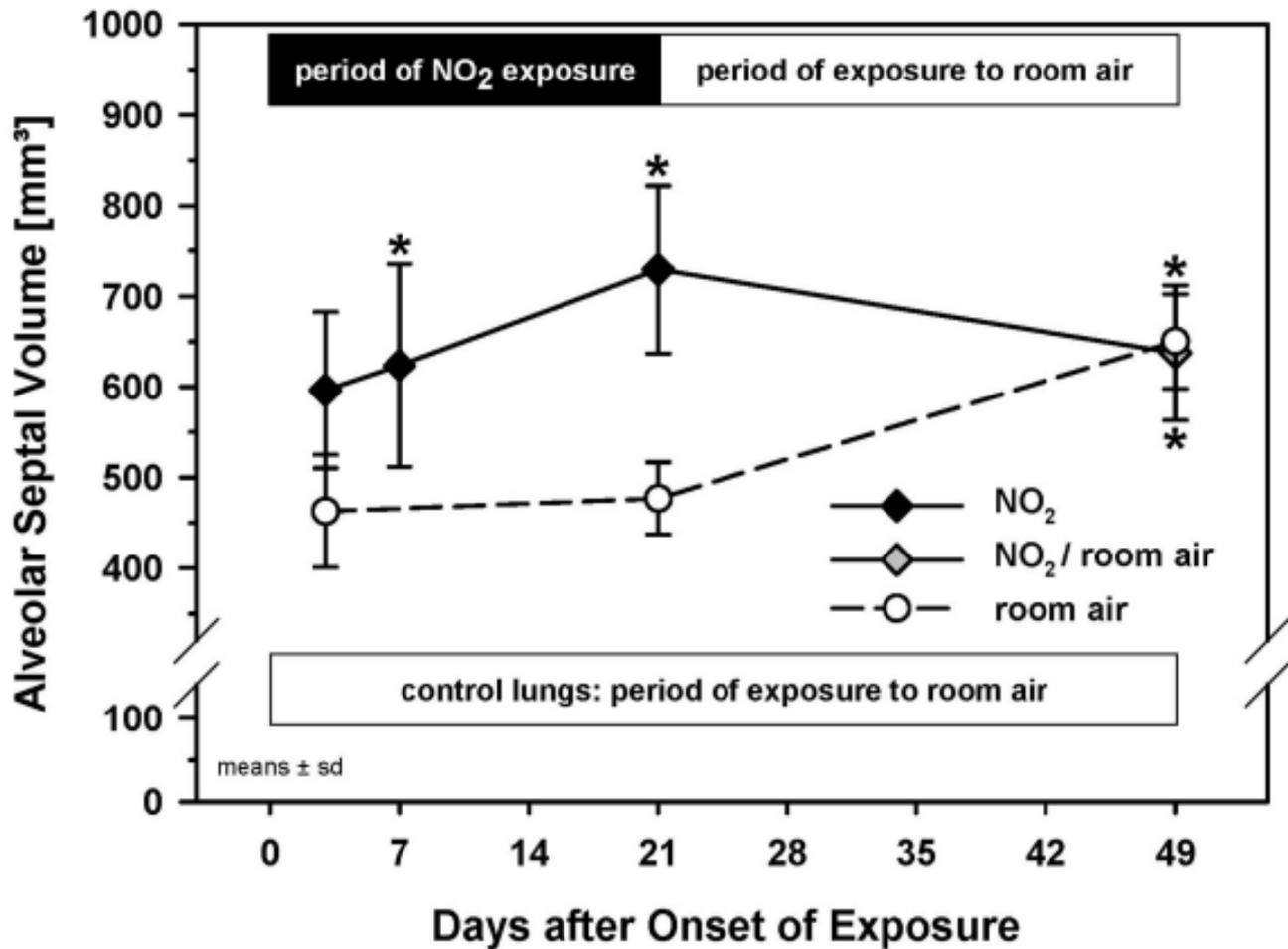
C

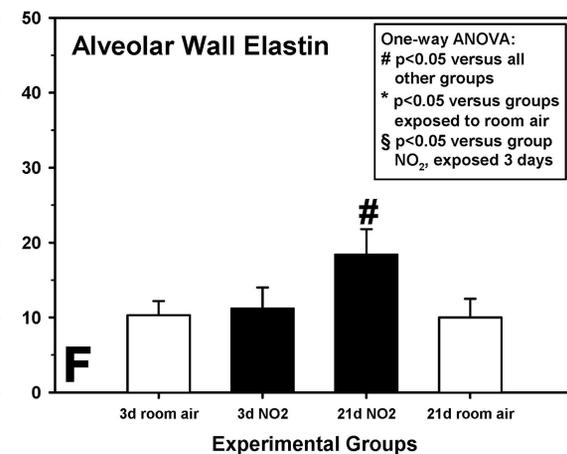
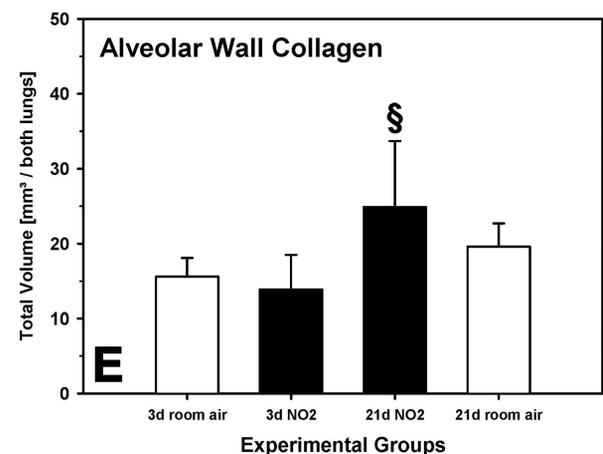
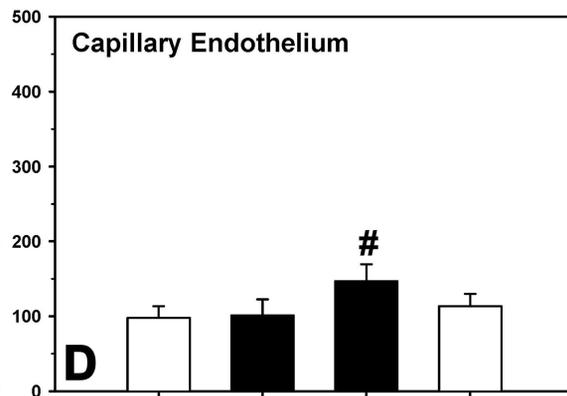
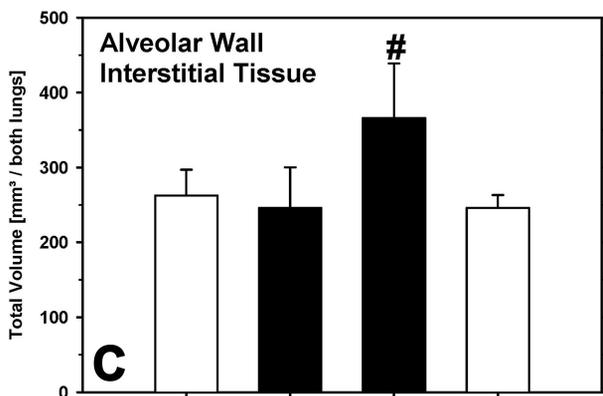
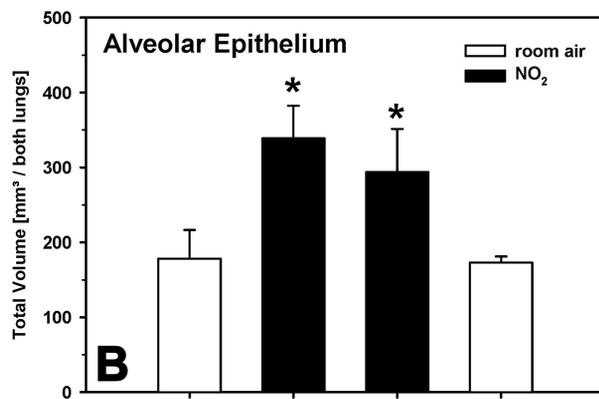
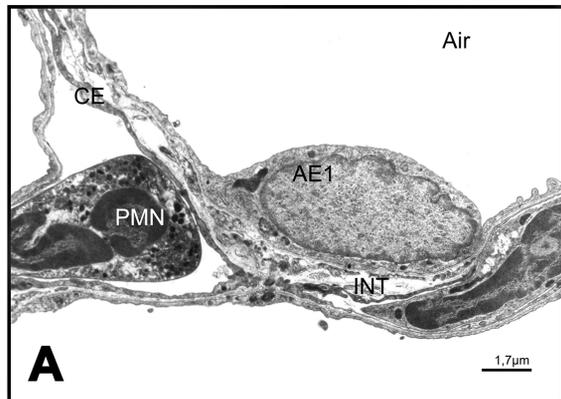


Exposure Time









One-way ANOVA:
 # p<0.05 versus all other groups
 * p<0.05 versus groups exposed to room air
 § p<0.05 versus group NO₂, exposed 3 days

