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EXPERIMENTAL

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Eight hours' inhalation of prostacyclin (PGI_2) in healthy lambs: effects on tracheal, bronchial, and alveolar morphology

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Abstract *Objective:* To study potential toxic effects of long-term (8 h) inhaled prostacyclin (PGI_2) on respiratory tract tissues.
Design: In a prospective, randomized order, either PGI_2 ($n = 7$) or normal saline ($n = 7$) was aerosolized during a time period of 8 h in healthy lambs.
Setting: Institute for Surgical Research of the Ludwig-Maximilians University of Munich.
Animals: 14 healthy, anesthetized, ventilated lambs.
Interventions: All animals were endotracheally intubated followed by tracheotomy. PGI_2 solution or normal saline was administered with a jet nebulizer (delivery rate 4–10 ml/h; mass median diameter of aerosol particles 3.1 μm).
Measurements and results: Histomorphological changes after 8-h inhalation of PGI_2 solution were compared to those after 8-h inhalation of normal saline. Tracheal and bronchoalveolar tissues were examined by light and electron microscopy in order to assess tissue damage induced by inhaled PGI_2. Pathological changes were ranked by a blinded observer following a graduation system ranging from "absence of pathological changes" to "maximal pathological changes". Abnormalities were restricted to the trachea (focal flattening of the epithelium, loss of cilia, slight inflammatory cell infiltration) and alveolar tissue (focal alveolar septal thickening with slight inflammatory cell infiltration), but no statistically significant differences between the PGI_2 and control groups were encountered.
Conclusion: Our findings indicate the absence of PGI_2 aerosol-related respiratory tissue damage after 8-h inhalation of PGI_2.

Key words Aerosols · Epoprostenol · Toxicity lung

Introduction

The reduction of increased pulmonary artery pressure may improve right ventricular function, decrease the effective pulmonary capillary filtration pressure and diminish the formation of pulmonary edema [1, 2]. Unfortunately, intravenous administration of potent vasodilators like prostacyclin (PGI_2) and prostaglandin E_1 (PGE_1) is hampered by their lack of selectivity for the pulmonary vasculature, resulting in a dose-dependent reduction of systemic vascular resistance. Furthermore, vasodilation in poorly ventilated areas of the impaired lung increases intrapulmonary shunt and worsens arterial oxygenation [3, 4].

Inhaled PGI_2 has been shown in several experimental [5, 6] and clinical studies [7–14] to dilate pulmonary vessels selectively, without a decrease in systemic vascular resistance. In these studies, the duration of inhalation never exceeded 1 h. Treatment of pulmonary hypertension with PGI_2 aerosol would, however, require repeated inhalation for longer period of time. The possible toxic

effects of inhaled PGI_2 on respiratory tract tissues, however, have not been investigated yet.

In a previous report, we demonstrated the absence of cardiorespiratory changes and the stability of platelet function after 8-h inhalation of PGI_2 in healthy lambs [15]. Important toxic effects on bronchoalveolar tissue were excluded by comparing the biochemical and cellular composition of the alveolar epithelial lining fluid before and after 8-h inhalation of PGI_2. Nevertheless, to exclude respiratory tissue damage requires the histomorphologic examination of respiratory tract tissue which were in contact with the inhaled agent.

Therefore, the continuation of the study mentioned above in healthy, anesthetized, ventilated lambs is reported, using light- and transmission electron-microscopical findings in tracheal and bronchoalveolar tissue after 8-h inhalation of prostacyclin aerosol.

Materials and methods

Animals

The study was performed in 15 healthy male and female lambs weighing between 28.5 and 48.5 kg. The lungs of all animals were histologically free of pneumonia. All animals received care in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1985). The study was approved by the local animal care and use committee.

Experimental groups

Fourteen animals were randomly assigned to two groups. In the PGI_2 group ($n = 7$), PGI_2 aerosol was administered over a time period of 8-h. In the control group ($n = 7$), 0.9% saline was aerosolized for the same time. One additional animal was intubated and killed after a short period of mechanical ventilation without inhalation of either PGI_2 or normal saline. The trachea and lungs of this animal were immediately fixed by the method described below.

Animal preparation

After intramuscular premedication with xylazine (0.1 mg/kg) and atropine sulfate (20 µg/kg), anesthesia was induced by intravenous injection of ketamine (5–8 mg/kg) and pancuronium bromide (0.25 mg/kg) and maintained by a continuous infusion of pentobarbital (5–10 mg/kg per h). Muscular paralysis was achieved by administering intravenous pancuronium bromide (0.1 mg/kg) every 2 h. Fluid losses were replaced by intravenous infusion of Ringer solution (15 ml/kg per h) throughout the experiment. Additional Ringer solution was infused, when necessary, to maintain pulmonary artery occlusion pressure at baseline level (median 10 mmHg). A warming pad was used to keep a minimum core body temperature above 37 °C. When the body temperature exceeded 40 °C, cooling was performed by positioning ice packs in the groins. During PGI_2 inhalation the animals were placed in the prone position.

All animals in the PGI_2 and control groups were first endotracheally intubated (Hi-Lo Jet, 10.0 mm i.d., Mallinckrodt, Argyle, N.Y., USA) and later tracheotomized (Hi-Lo Lanz, 10.0 mm i.d., Mallinckrodt Lab., Athlone, Ireland) to facilitate bronchoscopy, which was executed before and after 8-h inhalation of PGI_2 [15]. Mechanical ventilation was performed at a rate of 12 cycles/min with a fractional inspired oxygen (FIO_2) of 0.4 and positive end-expiratory pressure of 10 cmH_2O (Servo 900B, Siemens-Elema, Solna, Sweden). Inspiratory time was set to 1.66 s and tidal volume (V_T) was adjusted in order to maintain normocapnia. During inhalation, V_T and total inspiratory flow rate were maintained constant by reducing the inspiratory flow from the ventilator.

FIO_2 and end-expiratory CO_2-partial pressure were continuously monitored (Oxydig, Dräger AG, Lübeck, Germany; Nellcor N-2500, Nellcor, Hayward, Calif., USA). Peak and plateau airway pressure (P_{peak}, P_{plat}) were assessed using an electronic lung mechanics calculator (Model 940, Siemens-Elema, Solna, Sweden) connected to the ventilator.

Administration of PGI_2 aerosol

A jet nebulizer (Servo Nebulizer 945, Siemens, Germany) was connected to a Siemens 900B ventilator. A modified nebulizer chamber (Cirrus, Intersurgical, Twickenham, UK), which allowed refilling without disconnection of the nebulizer system, was attached to the endotracheal tube. The pressure supplied to the nebulizer chamber was monitored with an electronic manometer (Type 352-P, Debro, Meerbusch, Germany). The driving pressure of the nebulizer decreased during inspiration from 3.39±0.04 to 2.05±0.03 bar and, consequently, the flow rate of the nebulizer fell from about 15 to 9 l/min. Flow rates above 8 l/min were chosen to create particles mainly with a diameter of less than 2 µm, which are likely to settle in the alveolar region of the lung. Prior to the experiments, the particle size of the aerosol was measured by aerosolizing ^{99m}Tc-labeled PGI_2 solution. The chosen nebulizer and respirator settings used in the experiments produced aerosol particles with a mass median aerodynamic diameter of 3.1 µm (geometric standard deviation 1.9 µm).

PGI_2 was supplied as the sodium salt of epoprostenol (Flolan, Wellcome, London, UK) dissolved in 50 ml glycine buffer at pH 10.5. The solution was prepared on the day of the experiment and stored in the refrigerator until use. Before starting the experiment, the time period necessary for nebulizing 5 ml of 0.9% saline was noted. After extrapolation of this value, PGI_2 was diluted to obtain a target dose of 30 ng/kg per min. This resulted in a median dosage of 28 (20/36) ng/kg per min delivered to the animal over a time period of 8 h.

Efficacy of PGI_2 aerosol

In order to make sure that the PGI_2 aerosol actually reached distal airways in the present experimental set-up, efficacy of inhaled PGI_2 was studied in 3 additional animals (weight 32, 35, and 36 kg). For that purpose, the animals were ventilated with a hypoxic gas mixture (FIO_2 0.16) resulting in an increase of the pulmonary vascular resistance index (PVRI) of 15, 120, and 138%, respectively, compared to baseline values. Inhalation of PGI_2 aerosol in a dose of 19, 21, and 25 ng/kg per min reduced the increased PVRI by 23, 39, and 29%, respectively. In 2 animals, the systemic vascular resistance index (SVRI) increased by 8 and 9% during PGI_2 inhalation; in 1 animal SVRI decreased by 3%.

Assessment of morphological changes

The structural responses of the airways and peripheral airspaces of the animals to inhaled PGI_2 aerosol were studied using light and transmission electron microscopy. For this reason, lungs were fixed as reported below.

Fixation of the lung

After 8-h inhalation of PGI_2 or normal saline the lambs were placed in dorsal recumbency. The abdomen was opened and, after inducing cardiac arrest with a saturated potassium chloride solution, a bilateral pneumothorax was created by incision of both hemidiaphragms. The trachea and both lungs were filled with 2.5% glutaraldehyde in potassium phosphate buffer (pH 7.4, 350 mOsm) with a flow rate of 2.5 l/min. The fixative was left for 1 h at 20 cm water pressure [16, 17]. After in situ fixation, the trachea was clamped, the lungs were removed by thoracotomy and the left lung was submerged for 36 h in fresh fixative.

Preparation of the lung

Tissue samples for morphological analysis of the lung parenchyma and bronchial airways were taken from the cranial or caudal lobe of the left lung in a randomized order. The right lung, which was submitted two times to bronchoalveolar lavage during the experiment, was not used for histomorphological examination.

Lung parenchyma

The lung lobe was cut into slices 2-cm thick. From each of these sections tissue blocks were randomly chosen. The number of samples was based upon an area-weighted sampling method. This method consisted in placing a grid (distance of lines 0.5 cm) on the slices of parenchyma and taking every third cross point of lines as the sample area. More samples were taken from larger sections, resulting in between 12 and 20 samples per lung. For light microscopy, tissue blocks 5-mm thick were dehydrated in graded ethanol and embedded in paraffin. Sections 2–3 μm thick were stained with hematoxylin-eosin (overall number of sections: 20 to 40 sections per lung).

For transmission electron microscopy, tissue blocks 2-mm thick were postfixed in 2% osmium tetroxide, dehydrated in graded alcohols and propylene oxide and embedded in araldite resin. Sections 70- to 80-nm thick were made on a ultramicrotome (model HN 40, Jung, Mannheim, Germany) and were contrasted with uranyl acetate and lead citrate. Preparations were examined in Zeiss EM 900 electron microscope (Zeiss, Jena, Germany).

Trachea and bronchial airways

At 1 cm above the tracheal bifurcation a 5-mm thick ring was cut and dissected into four quadrants. Bronchial airways were cut by the microdissection method [18] and airways of the generation numbers 5, 10, and 15 were examined morphologically by light and transmission electron microscopy. Embedding, staining, and examination methods were identical to those described above. Each tissue sample gave to 3 to 5 sections.

Histomorphological examinations

Tissue injury determined by light microscopy was scored by a blinded pathologist. Score 0 corresponded to the absence of abnormalities, 1 to minimal damage, 2 to mild damage, 3 to moderate damage, 4 to severe damage, and 5 to maximal damage, according to assessments of polymorphonuclear infiltration of airspace or vessel walls, flattening of respiratory epithelium, loss of cilia, hyaline membrane formation, hemorrhage in the epithelial layer and subepithelial region, thickness of alveolar wall, alveolar congestion, and edema. If there were normal and abnormal regions in the same microscopical section, only the score for the most abnormal region was entered into the statistical evaluation of the section.

A second blinded pathologist examined 960 alveolar sections from seven lambs (three PGI_2, three control animals, and the short-time ventilated animal) by transmission electron microscopy. Per section, 20 to 25 areas were examined. The examination was limited to description of morphological changes of the alveolar region. Morphometric studies were not undertaken.

Statistics

For statistical analyses the SAS software package (version 6.08; SAS Institute, Cary, N.C., USA) was used. Shapiro-Wilk's statistic indicated non-normal distribution of data, therefore non-parametric tests were used. Results are presented as median and first and third quartiles.

For within-group analysis, Friedman's test was used to assess the effect of time in each group. Significant *F*-values led to Wilcoxon's signed rank testing for the timepoints "before" and "after 8-h inhalation". For between-group analysis, analysis of variance of ranked data was used for assessing differences between groups. For significant *F*-values, the Mann-Whitney U-test was used to detect differences "before" and "after 8-h inhalation". Analysis of variance and Friedman's test were used prior to testing individual measurements in order to avoid increasing the alpha error by multiple testing [18]. Histological damage-scoring values were tested directly by the Mann-Whitney U-test to detect differences between groups.

Results

P_{peak} and P_{plat} airway pressures

In the control group, median P_{peak} was 27 (23/31) cmH_2O before inhalation compared to 26 (24/27) cmH_2O after 8-h inhalation of normal saline; in the PGI_2 group, P_{peak} was 26 (22/28) cmH_2O and 26 (24/30) cmH_2O, respectively. In the control group, median P_{plat} was 25 (22/27) cmH_2O before inhalation and 26 (23/27) cmH_2O after 8-h inhalation of normal saline. In the PGI_2 group, median P_{plat} was 23 (18/27) cmH_2O before and 25 (23/29) cmH_2O after 8-h inhalation. No statistically significant differences were detected within or between the two groups.

Light microscopy

The results of histological damage scoring by light microscopy are listed in Table 1. In the PGI_2 group and

Table 1 Tissue-damage scoring[a] performed by a blinded pathologist. Values are medians (1st quartile/3rd quartile). *G5* bronchus of the fifth generation, *G10* bronchus of the tenth generation, *G15* bronchus of the fifteenth generation)

Group	Trachea	Bronchus (G5)	Bronchus (G10)	Bronchus (G15)	Alveoli
NaCl ($n = 7$)	1 (1/4)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/1)
PGI_2 ($n = 7$)	1 (1/3)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/1)

[a] 0 = no abnormalities; 1 = minimal damage; 2 = mild damage; 3 = moderate damage; 4 = severe damage; 5 = maximal damage

in the NaCl group, 57% of tracheal sections (16 of 28 sections) showed pathological alterations (maximal damage scores: PGI_2 group: 4; NaCl group: 5). In both animal groups they consisted mainly of focal flattening of tracheal epithelium with focal loss of cilia (Fig. 1). Rarely, slight inflammatory cell infiltration (polymorphonuclear and mononuclear cells) in the epithelial layer and the subepithelial region was observed (Fig. 2).

Four percent of bronchial sections of each generation and each group were altered (slight inflammatory cell infiltration in the epithelial layer and the subepithelial region; maximal damage score: 1). Seven percent of alveolar sections were altered in the PGI_2 group (maximal damage score: 3), 12% in the NaCl group (maximal damage score: 3). Changes consisted in thickening of the alveolar septal space and focal inflammatory cell infiltration (Fig. 3).

There were no statistically significant differences between the two groups for tracheal, bronchial, and alveolar tissue damage. In the short-term ventilated animal no alterations were detected in tracheal and bronchial sections; 8% of alveolar sections were altered (thickening of the alveolar septal space and focal inflammatory cell infiltration; maximal damage score: 1).

Electron microscopy

Overall, 960 sections of the alveolar region were evaluated by transmission electron microscopy. Swelling of type-I pneumocytes was detected in 2 sections of the PGI_2 group (50% of the areas examined), 3 sections of the NaCl group (35% of the examined areas), and in 4 sections of the control animal (20% of the examined areas). In the short-term ventilated animal, swelling was distinctly less pronounced. Otherwise, the alveolar-capillary complex, particularly basal membranes and capillary endothelium, were intact. Type II pneumocytes as well as alveolar macrophages showed not pathological abnormalities (Figs. 4–6).

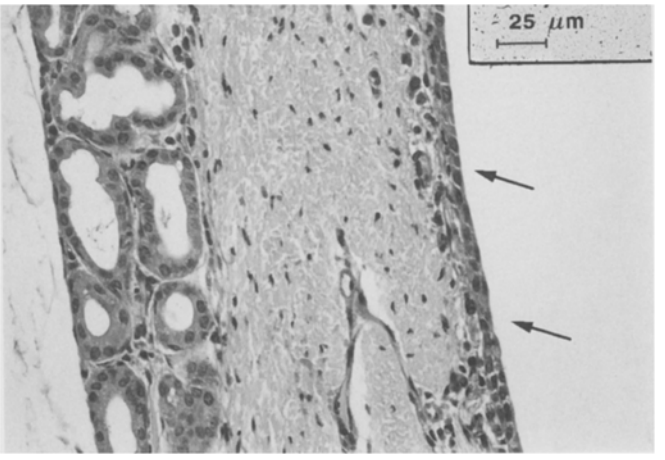

Fig. 1 Cross-section of the tracheal mucosa after 8-h inhalation of PGI_2 aerosol: flattening of respiratory epithelium and loss of cilia (arrows). Original magnification ×100

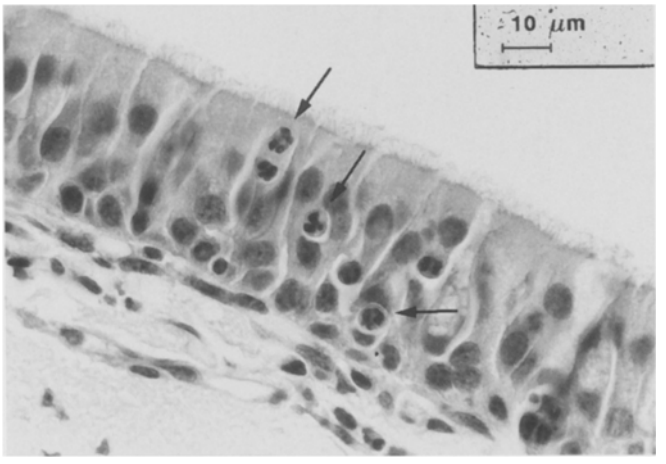

Fig. 2 Cross-section of the tracheal mucosa after 8-h inhalation of PGI_2 aerosol: epithelial infiltration with polymorphonuclear cells (arrows). Original magnification ×250

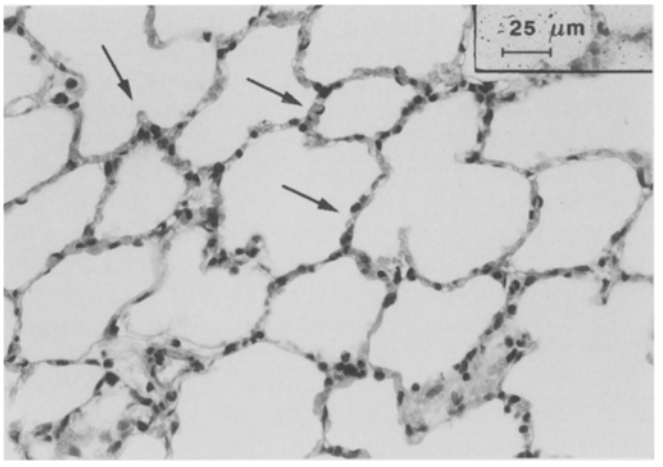

Fig. 3 Cross-section of alveolar epithelium after 8-h inhalation of PGI_2 aerosol: septal thickening and infiltration with polymorphonuclear cells (arrows). Original magnification ×100

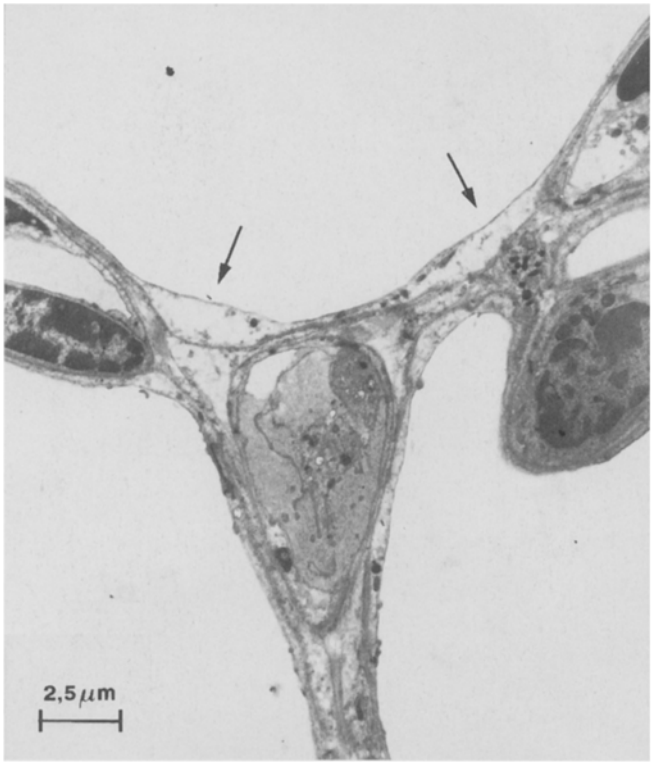

Fig. 4 Transmission electron micrograph of alveolar epithelium after 8-h inhalation of prostacyclin aerosol: swelling of type-I pneumocyte (arrows). Original magnification ×3000

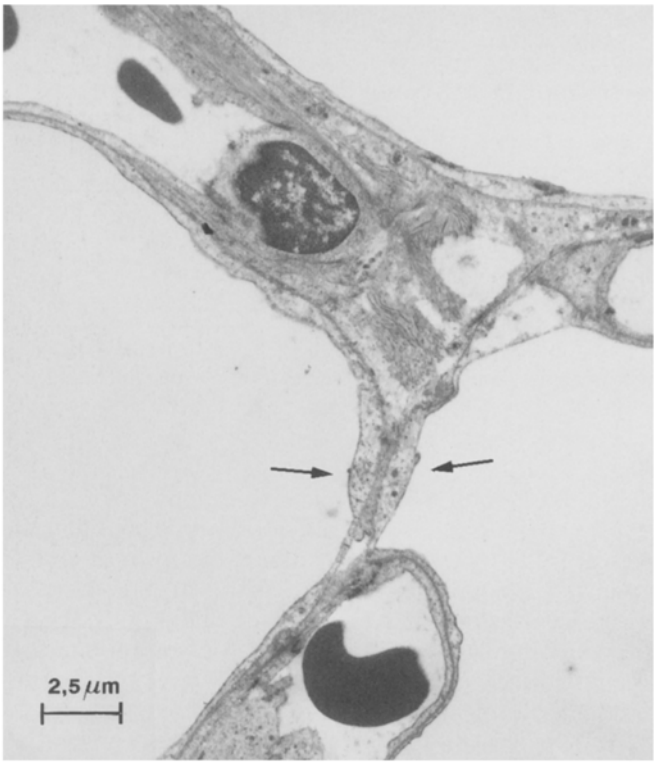

Fig. 5 Transmission electron micrograph of alveolar epithelium after 8-h inhalation of normal saline: swelling of type-I pneumocyte (arrows). Original magnification ×3000

Discussion

The objective of our study was to identify potential morphological changes after prolonged inhalation (8 h) of PGI_2. We used healthy lambs to ensure that changes observed during a prolonged inhalation were not confounded by underlying disease or by the experimental pulmonary injury.

For our study, we chose a median PGI_2 dose of 28 (20/36) ng/kg per min, corresponding to a total dose of 500 (320/650) µg of PGI_2 administered over 8 h. Compared to doses known to reduce pulmonary hypertension in patients (2–50 ng/kg per min), this represents a medium dose. The pulmonary deposition fraction of inhaled prostacyclin has not been directly assessed in our experiments. Nevertheless in three animals with hypoxia-induced pulmonary hypertension, increased PVRI was selectively reduced by inhaled PGI_2 aerosol, allowing the assumption that in our experiments a sufficient amount of PGI_2 must have reached the alveoli.

The first histomorphological changes of respiratory tract tissue have been shown to appear 15 min after exposition to the inhaled toxic agent. In a sheep model of smoke inhalation injury [19], the most significant changes consisted in necrosis and sloughing of respiratory tract epithelium, less significant changes in swelling of the respiratory epithelium, loss of cilia, and surface erosions.

Inflammatory respond, manifested grossly by the formation of pseudomembranes (typically in major airways), was observed 2 h after starting smoke inhalation. Simultaneously, neutrophils appeared in the lamina propria, epithelium, and airway lumen of trachea and bronchi.

Proceeding from these initial observations, pathological changes after 8-h inhalation of prostacyclin, if they existing, should have been detected.

The region primarily affected by pathological change caused by an inhaled toxic substance is the trachea. Fuller et al. [20] demonstrated that only 1.2–2.9% of an aerosol dose was actually deposited in the lung. A large fraction of aerosol accumulated in the conducting airways, particularly the trachea and endotracheal tube. In fact, the most significant lesions after smoke inhalation were always most severe in the tissue adjacent to the tip of the endotracheal tube.

Analogous to these findings, the most distinct histomorphological abnormalities in our experiments were also seen in the tracheal sections. We saw slight focal

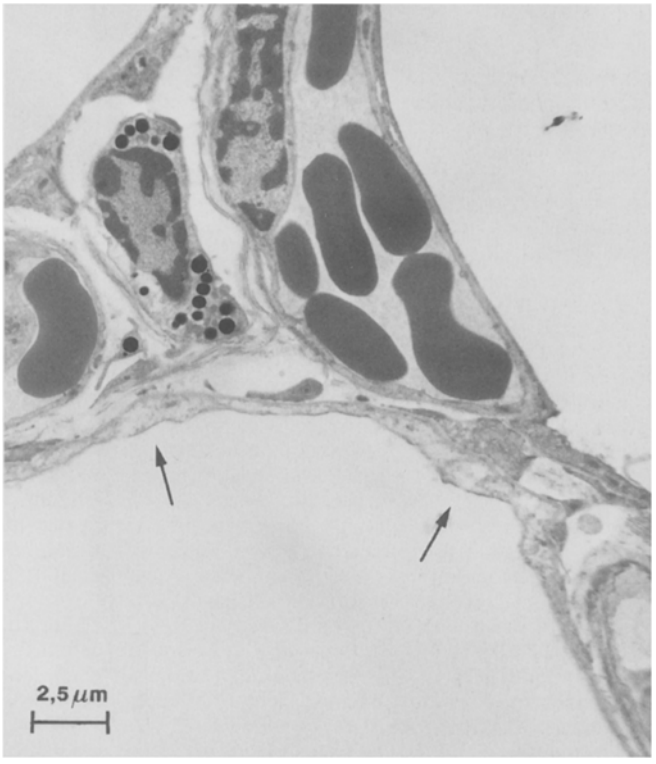

Fig. 6 Transmission electron micrograph of alveolar epithelium after short-time ventilation. Original magnification ×3000

flattening of the respiratory epithelium, focal loss of cilia, and slight inflammatory cell infiltration in the epithelium and the subepithelial layer. Since these changes were observed in both the PGI_2 group and the control group, they cannot be considered to be prostacyclin-related. In one additional lamb, which was intubated and ventilated only for a few minutes and which underwent no bronchoalveolar lavage, these morphological changes of the trachea could not be detected. The findings suggest that the observed abnormalities in the tracheal sections were most likely caused by mechanical irritation of the respiratory epithelium upon exposure to the bronchoscope, rather than being caused by the aerosols studied.

The detection of slight alveolar epithelial damage due to an inhaled substance calls for ultrastructural examination. Target cells for the toxic effect of an inhaled substance are type-I pneumocytes. In the study of Hubbard et al. [19], mild swelling of type-I cells and changes in the membrane-bound vacuoles in type-II cells were found. Additionally, there was mild interstitial edema with septal thickening.

In the present study, similar changes in type-I cells and focal alveolar septal thickening were detected, but again these changes were present in the PGI_2 group as well as in the control group. Merely in the single, short-term ventilated lamb these changes were less prominent. These findings suggest that the pathological changes are primarily due to the effects of mechanical ventilation and are not related to the substance inhaled.

Median P_{peak} and P_{plat} airway pressures never exceeded 30 cmH_2O in our experiments. Webb and Tierney [21] showed, however, that ventilation of rats over a time period of 60 min with peak inspiratory pressures of 30 cmH_2O resulted in interstitial pulmonary edema.

John et al. [22] reported abnormalities of type-I pneumocytes, similar to those found in our electron-microscopical examination, after 6-h mechanical ventilation of rabbits with humidified air. All animals were ventilated by means of a pressure-controlled, flow-regulated ventilator, and peak airway pressure was maintained at 20 cmH_2O. Therefore, high inspiratory peak airway pressures in combination with long-term exposure of the lambs to mechanical ventilation may explain the ultrastructural changes observed in our experiment.

In summary, the histomorphological abnormalities in tracheal, bronchial and alveolar epithelial tissues after 8-h inhalation of PGI_2 in healthy lambs do not seem to be related to the substance inhaled. Changes in tracheal tissues are most likely due to mechanical irritation of the epithelium by bronchoscopy and the slight alveolar alterations to effects of mechanical ventilation.

We, therefore, conclude that 8-h inhalation of PGI_2 aerosol does not induce histopathological changes in respiratory tissues. Inhalation of PGI_2 over this time period therefore appears safe in lambs and might be applied without harm to patients.

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