Short-term exposure to high-pressure ventilation leads to pulmonary biotrauma and systemic inflammation in the rat

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Abstract. Though often lifesaving, mechanical ventilation itself bears the risk of lung damage [ventilator-induced lung injury (VILI)]. The underlying molecular mechanisms have not been fully elucidated, but stress-induced mediators seem to play an important role in biotrauma related to VILI. Our purpose was to evaluate an animal model of VILI that allows the observation of pathophysiologic changes along with parameters of biotrauma. For VILI induction, rats (n=16) were ventilated with a peak airway pressure (p_{max}) of 45 cm H_2O and end-expiratory pressure (PEEP) of 0 for 20 min, followed by an observation time of 4 h. In the control group (n=8) the animals were ventilated with a p_{max} of 20 cm H_2O and PEEP of 4. High-pressure ventilation resulted in an increase in $paCO_2$ and a decrease in paO_2 and mean arterial pressure. Only 4 animals out of 16 survived 4 h and VILI lungs showed severe macroscopic and microscopic damage, oedema and neutrophil influx. High-pressure ventilation increased the cytokine levels of macrophage inflammatory protein-2 and IL-1ß in bronchoalveolar lavage and plasma. VILI also induced pulmonary heat shock protein-70 expression and the activity of matrix metalloproteinases. The animal model used enabled us to observe the effect of high-pressure ventilation on mortality, lung damage/function and biotrauma. Thus, by combining barotrauma with biotrauma, this animal model may be suitable for studying therapeutical approaches to VILI.

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Introduction

The application of mechanical ventilation is a key factor in the treatment of critically ill patients. However, mechanical ventilation itself can cause adverse effects on lung parenchyma and gas exchange, resulting in ventilator-induced lung injury (VILI). In patients with severe respiratory failure in particular, high airway pressures can aggravate structural lung damage and increase systemic cytokine levels and mortality rates (1).

To further improve the outcome of critically ill patients, a better understanding of the detrimental mechanisms of VILI is therefore required. In 1974, Webb and Tierney were the first to demonstrate that high peak airway pressures result in lung oedema, alveolar disruption, capillary leakage and death (2). In addition to this direct lung damage by volu- and/or barotrauma, studies performed subsequently highlighted the significance of local inflammation and lung-borne cytokines (biotrauma). This inflammatory reaction is not confined to the lungs but also extends to systemic circulation, thus contributing to the development of systemic inflammatory response (SIRS) and multiple organ dysfunction syndrome (MODS) (3).

However, the role of proinflammatory mediators in the course of VILI is still controversial, as several *in vitro* and *in vivo* models of VILI have shown inconsistencies in cytokine response (4). Most studies focusing on cytokine release during VILI were performed in pre-damaged lungs. In these so-called 'two-hit models', in addition to mechanical injury the animals were exposed to surfactant depletion (5), hyperoxia (6) or endotoxemia (7).

Therefore, in order to isolate the specific inflammatory effects of mechanical stress, we established an animal model of ventilator-induced lung injury in previously healthy rats. In addition to the pathophysiologic changes in terms of barotrauma, we aimed to characterize the local and systemic inflammatory response to VILI in terms of biotrauma. For the investigation of possible therapeutic agents, an animal model of VILI that allows the simultaneous observation of local and systemic inflammation, impairment of lung structure and function and, above all, systemic alterations and mortality is of special interest.

Materials and methods

Animal preparation. All animal experiments were approved by the governmental board for the care and use of animal subjects (Regierungspraesidium Darmstadt, Germany) and performed according to the 'Guide for the Care and Use of Laboratory Animals', National Academic Press, Washington D.C., 1996. Male Sprague-Dawley rats (n=24) were obtained from Janvier (Le Genest-St-Isle, France; mean body weight ± SD, 600±30 g). Rats were anesthetized initially by i.p. injection of pentobarbital (Narcoren, Merial, Halbergmoos, Germany; 50 mg/kg) and fentanyl (Janssen-Cilag, Neuss, Germany; 0.05 mg/kg), weighed and then placed supine on a heating pad. Animals were tracheotomised and ventilated with an infant ventilator (Stephanie®, Stephan, Gackenbach, Germany) using pressure controlled ventilation, with the following ventilator adjustments as the standard: peak airway pressure (p_{max}), 20 cm H_2O; end-expiratory pressure (PEEP), 4 cm H_2O; f, 30/min; fraction of inspired oxygen (FiO_2), 0.21; inspiratory to expiratory time ratio (I:E), 1:2. A temperature probe was inserted rectally for the continuous monitoring of body temperature in order to keep it constant at 37.0-38.0°C throughout the experiment. Fluid-filled polyurethane catheters (ID, 0.58 mm; OD 0.96, mm; SIMS Portex Ltd, Hythe, UK) were inserted in the right femoral vein and artery for the infusion of anaesthetics and blood gas analysis, respectively. Anaesthesia was maintained by the continuous i.v. infusion of pentobarbital (5-10 mg/kg/h) and fentanyl (2.5-5.0 μg/kg/h).

Experimental groups. After a stabilization period of 15 min, rats were randomly assigned either to the injurious mechanical ventilation group (highP; n=16) or the control group (lowP; n=8). For the induction of VILI in the highP group, the ventilator setting was changed for 20 min to a p_{max} of 45 cm H_2O, PEEP of 0 cm H_2O and I:E of 1:1, and afterwards reset to the standard ventilator adjustments. The control group (lowP) was ventilated with standard ventilator adjustments for the observation time of 4 h.

Rats were monitored for 4 h and hourly blood gas analyses were performed. At the end of the protocol animals were exsanguinated and blood samples were obtained. After thoracotomy and inspection of the lung the accessory lobe was ligated and the rest of the lung was lavaged with PBS (Serva, Heidelberg, Germany). Subsequently, the accessory lobe was cut into three parts, one part was fixed in 10% buffered formalin for histologic examination and the other two were snap-frozen in liquid nitrogen and stored at -80°C.

This study focused on the evolving inflammatory processes. Therefore, no plasma, bronchoalveolar lavage (BAL) or lung probes were harvested in animals that died before the critical time period of 2 h after the induction of VILI. In this context, Chiumello *et al* (8) showed that after the beginning of injurious ventilation, local and systemic levels of proinflammatory cytokines increase over time, with a high peak between 2 and 4 h.

Macroscopic lung injury score. We assessed the macroscopic lung damage according to the classification described by Lim *et al* (9). Therefore, both lung lobes were divided on the ventral and dorsal surface into upper and lower halves and the presence and extent of haemorrhage was examined. When more than half of the lung surface showed haemorrhage this was correlated with a score of 2, less than half with a score of 1 and no visible severe lung damage with a score of 0. This resulted in a lung injury score from 0 (uninjured lung) to 16 (maximal lung damage).

Isolation of BAL cells, BALF and lung tissue. The broncho-alveolar lavage fluid obtained was centrifuged and the supernatant (BALF) was frozen and stored at -80°C. The cell suspension was used for a differential cell count according to morphologic criteria in cytocentrifuged smears stained with May-Gruenwald-Giemsa. To analyze nitrite release as a read-out for inducible nitric oxide synthase (iNOS) activation, cells were seeded on polystyrene plates, and after initial cultivation for 2 h non-adherent cells were removed by washing. Adherent cells were regarded as alveolar macrophages and incubated at 37°C in 5% CO_2 for 20 h. Nitrite concentrations in cell culture supernatants were determined by Griess assay (10).

For Western blotting, lung samples were homogenized in lysis buffer (150 mM NaCl, 1 mM $CaCl_2$, 25 mM TrisCl, pH 7.4, 1% Triton X-100), supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), DTT, Na_3VO_4, PMSF (each 1 mM) and NaF (20 mM). The tissue extract was cleared by centrifugation and the supernatant was frozen and stored at -80°C. Total protein concentrations of BALF samples and lung homogenates were determined with the Bradford assay (11).

Plasma samples and ELISA assay. The blood samples were centrifuged and the resulting plasma was collected and frozen at -80°C. The BALF and plasma levels of macrophage inflammatory protein-(MIP)-2 (Biosource, Camarillo, CA) and IL-1ß (R&D Systems, Wiesbaden, Germany) were determined by ELISA according to the manufacturer's instructions.

Western blot analysis and SDS-PAGE zymography. Western blot analysis of heat shock protein (HSP)-70 (StressGen, Ann Arbor, MI) in lung homogenates was performed using standard procedures.

Gelatinolytic activity of proteins from BALF (30 μl) was assessed as described previously (12). Gelatinolytic activity of proteins was visualized as lightened areas on an otherwise blue gel and their migration properties were determined by comparison with prestained full-range rainbow protein markers (Amersham Pharmacia Biotech, Freiburg, Germany).

Statistics. Data are presented as median ± semi-interquartile range (IQR). Survival in the two study groups was compared using a log-rank test and differences between the groups were analyzed using the Mann-Whitney U test (Sigma Stat 3.1, San Jandel, CA). Statistical significance was accepted at p<0.05.

Results

Outcome and physiopathological evaluations. In the lowP group all animals (n=8) survived the observation period of

Table I. Mean arterial pressure (MAP), pH and tidal volume (V_T) over time.

	Baseline	1 h	2 h	3 h	4 h
MAP					
lowP	137.0 (5.4)	123.0 (12.0)	112.5 (7.4)	115.0 (12.6)	119.5 (9.8)
highP	147.0 (7.4)	106.5 (28.3)	47.5 (19.4)[a]	76.0 (13.9)	62.5 (6.3)[a]
pH					
lowP	7.48 (0.03)	7.45 (0.03)	7.43 (0.03)	7.40 (0.02)	7.39 (0.03)
highP	7.43 (0.07)	7.18 (0.04)[a]	7.24 (0.16)[a]	7.23 (0.07)[a]	7.23 (0.04)[a]
V_T					
lowP	8.3 (0.4)	8.3 (1.0)	8.3 (1.1)	8.3 (1.2)	7.9 (1.6)
highP	8.9 (0.6)	4.5 (0.4)[a]	3.9 (0.5)[a]	4.1 (0.8)[a]	4.1 (0.8)[a]

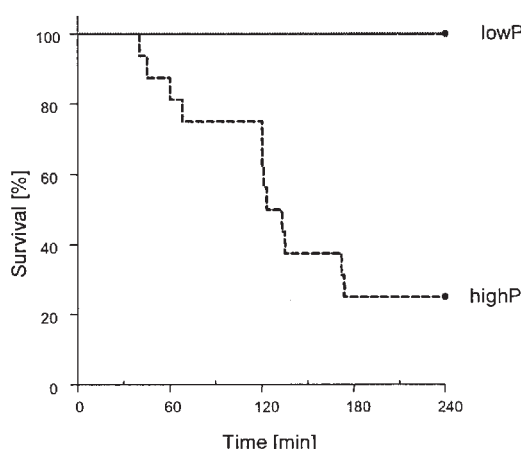Values shown as median (semi-IQR); [a]$p < 0.05$ versus lowP.

Figure 1. Kaplan-Meier survival curves for lowP and highP groups. All animals ventilated with the low-pressure regime (lowP, n=8) survived the observation time of 240 min, whereas only 4 animals of the highP group (20 min of ventilation with a peak airway pressure of 45 cm H_2O; n=16) were still alive at this time point (log-rank test, $p < 0.001$).

Table II. Bronchoalveolar lavage (BAL) characteristics and differential cell count.

	lowP	highP
Macrophages ($x10^4$/ml)	446 (68)	142 (45)[a]
Neutrophils ($x10^4$/ml)	13 (8)	84 (38)[a]
Protein content (μg/ml)	249 (15)	1451 (270)[a]

Values shown as median (semi-IQR); [a]$p < 0.05$ vs. lowP.

4 h. In the highP group only 4 animals of 16 survived 4 h (Fig. 1). For analysis of gas exchange, haemodynamics, tidal volume (V_T) and macroscopic lung injury score, all animals of the highP group were included. As mentioned above, for further analysis of the inflammatory response after VILI only the animals that survived at least 2 h after VILI induction were included.

In the highP group paO_2 declined and $paCO_2$ rose over time whereas in the lowP group both parameters remained stable. There was a significant difference at time points 1-4 h after VILI induction between the two groups (Fig. 2).

Mean arterial pressure declined over time in the highP group and was significant lower compared to the lowP group at time points 2 and 4 h. Animals became acedotic in the highP group over time and pH was significantly lower 1 to 4 h after VILI induction compared to the lowP group. Tidal volume declined after high-pressure ventilation and was found reduced to 50% when compared to low-pressure ventilation (Table I).

After 20 min of injurious ventilation, the lungs of all the highP rats showed severe macroscopic damage (Fig. 3A). As compared to the lungs from the lowP group, they appeared enlarged, swollen and diffusely haemorrhagic (Fig. 3B).

The lungs of the lowP animals showed no haemorrhage (i.e. lung injury score, 0), while the lung injury score of the highP group was 11.0 (3.25) [median (semi-IQR), $p < 0.001$].

Histological examination of lungs ventilated with low pressure showed normal architecture (Fig. 3C) whereas lungs exposed to high-pressure ventilation showed massive interstitial and alveolar cell infiltration, oedema and haemorrhage (Fig. 3D). Overall the highP lungs showed severe deterioration with atelectasis and consolidation of lung tissue.

BAL characteristics. Total fluid volumes recovered by BAL were 75.0 (0.8) ml (lowP group) and 68.0 (3.0) ml (highP group), respectively (p=0.002). High-pressure ventilation shifted the neutrophil/macrophage ratio towards the neutrophils and caused an increase in BAL protein content (Table II). Overall, fewer cells were lavageable in the highP group. Compared with low-pressure ventilated animals, a markedly increased nitrite accumulation [5.9 (1.7) vs. 28.6 (15.8) nmol/20 h] was detected in the supernatants of cultured alveolar macrophages from the highP group (Fig. 4).

Parameters of inflammation. High-pressure ventilation augmented the MIP-2 and IL-1ß concentrations both in BAL and plasma as analyzed by ELISA (Fig. 5).
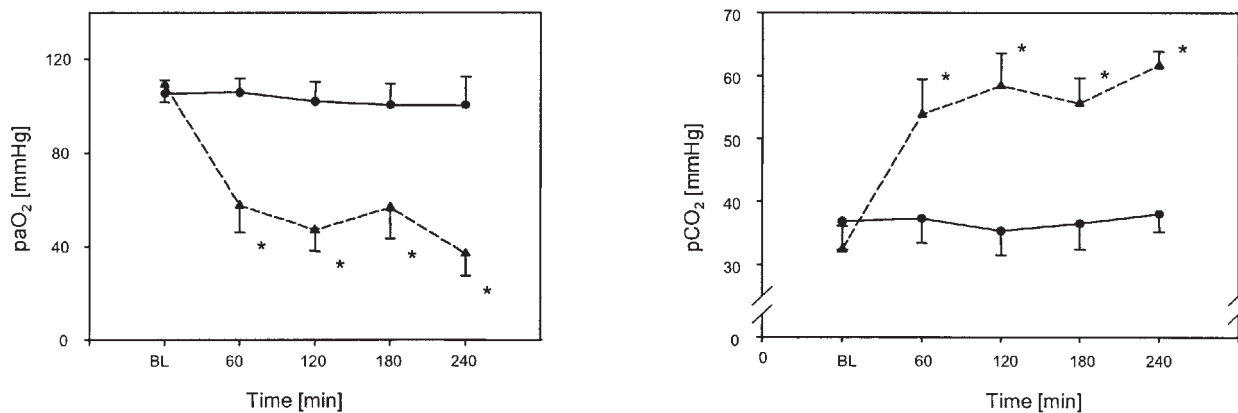
Figure 2. Trend of arterial partial pressure of oxygen (paO_2) and carbon dioxide ($paCO_2$) over time. Solid circles, low pressure (lowP) group; solid triangle, high pressure (highP) group. Median (semi-IQR); *p<0.05 vs. lowP group.
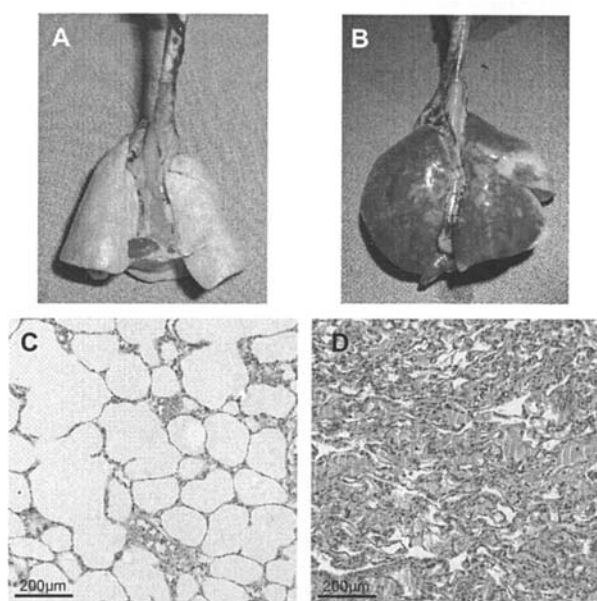

Figure 3. Macroscopic and microscopic appearance of the lungs of representative control animals (lowP) (A and C), and animals after 20 min of high-pressure ventilation (p_{max}, 45 cm H_2O) (highP) (B and D). Massive pulmonary haemmorhage/congestion can be seen in the highP lungs. Lungs were inflated with the appropriate tidal volume. (C) Haematoxylin and eosin-stained lung tissue sections (magnification x200) showing normal lung architecture in the lowP group. (D) Lung histology in the highP group characterized by perivascular and interstitial edema, inflammatory cell infiltration, intra-alveolar haemhorrhage, atelectasis and disorganisation of lung architecture.

Figure 4. Nitrite release from cultured alveolar macrophages (0.2×10^6/well) in 20 h. Median (semi-IQR); *p<0.05 vs. lowP.

Western blot analysis showed that HSP-70 expression in lung homogenates in the highP group was higher than in the lowP group (Fig. 6).

The zymogram of the BALF in animals ventilated with low pressure displayed weak gelatinolytic bands for the latent forms of gelatinase A (proMMP-2) and gelatinase B (proMMP-9). In contrast, BALF from animals ventilated with high pressure displayed intense gelatinolytic bands of activity for the matrix metalloproteinases in their latent and active forms (proMMP/MMP-9 and proMMP-2/MMP-2) (Fig. 7).
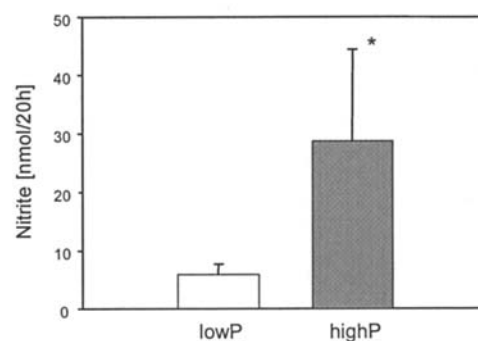
Discussion

In the present study, short-term high-pressure ventilation (20 min of p_{max}, 45 cm H_2O and PEEP, 0) in healthy rats resulted in severe ventilator-induced lung injury. Animals in the high-pressure group showed a 4-h mortality of 75%, impairment of gas exchange and haemodynamics and massive destruction of lung architecture. In addition, the mechanical stress resulted in an activation of neutrophils and alveolar macrophages, in an increase of local and systemic proinflammatory cytokines and in the induction of the pulmonary expression of HSP-70 and MMP-9 and -2.

Ventilation with a peak inspiratory pressure of 45 cm H_2O for 20 min has been used in other studies to induce VILI (2,13,14). However, these studies only focused on physiological parameters such as blood gases and/or blood pressure and structural damage to the lung leading to the alteration of respiratory mechanics and destruction of the alveolocapillary border. Our study adds to the present knowledge the characterization of biotrauma parameters, showing an influx of inflammatory cells into the lung, local and systemic release of proinflammatory cytokines, and activation of the cellular stress response.

The lungs in the highP group were grossly haemorrhagic, congested and markedly enlarged; in some animals the tracheal cannula was completely filled with oedema fluid.
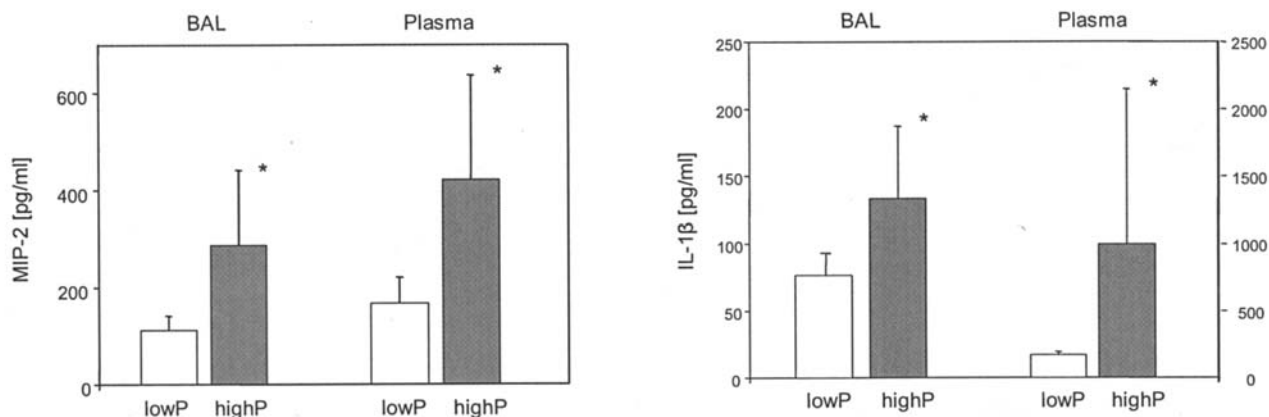
Figure 5. Plasma and bronchoalveolar lavage (BAL) cytokine levels of MIP-2 and IL-1ß. Median (semi-IQR); *p<0.05 vs. lowP.
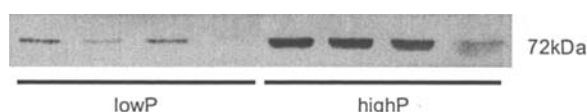

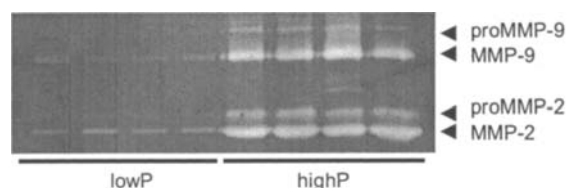Figure 6. HSP-70 detection in lung homogenates by Western blot analysis; 4 animals per group.

Figure 7. Gelatine zymography of bronchoalveolar lavage (BAL) specimens from 4 animals of the lowP and highP group, respectively.

This ventilator-induced pulmonary oedema is associated with severe endothelial and epithelial abnormalities, the structural counterpart of alterations in permeability (2). With reference to this, light microscopy showed perivascular oedema, intra-alveolar haemorrhage and disorganization of lung structure in the highP group, which is pathognomonic for severe lung injury (15). Furthermore the protein content in the BAL (as a marker of barrier disruption), was elevated after injurious ventilation. The rapid evolution of pulmonary oedema and atelectasis may be the reason that the V_T after 20 min of high-pressure ventilation was only half the V_T at baseline conditions.

The destruction of the alveolo-capillary unit resulted in the impairment of lung function with hypoxemia, hypercapnia and acidosis. Moreover, the decline of the systemic blood pressure resulted in global and local hypoperfusion, which may have also contributed to the worsening of the arterial blood gases.

Another typical finding in lung inflammation models is the decrease in BAL macrophage and the increase in BAL neutrophil counts (16). It is assumed that an increased adherence of the alveolar macrophages to alveolar epithelium may make them less easily recoverable by lavage. Whitehead et al (16) showed that after high tidal volume ventilation the number of interstitial macrophages was greater than that in the alveolar compartment. In our study the influx of neutrophils could not only be seen in the BAL of highP animals but also in the light microscopy of lung specimens.

The most important chemotactic stimuli that recruits neutrophils from the blood to the pulmonary site is interleukin-8 (17). The concentration of MIP-2 (the equivalent of IL-8 in the rat) was found to be significantly elevated in the BAL and plasma of highP animals. This increase in MIP-2 concentration in BAL and plasma has already been noted in various models of endotoxin- (18,19) and ventilator-induced (8,20) lung injury. Whether high-pressure ventilation per se can cause a significant release of proinflammatory cytokines is still controversial. Several studies failed to show an increase in TNF-α or IL-1ß concentration in BAL or plasma after injurious ventilation strategies (21,22). On the other hand, there are VILI studies that, as in our study, were able to detect elevated levels of proinflammatory cytokines in BAL and plasma (23-25). Moreover, pre-injured ('primed') lungs appear to be more susceptible to the development of VILI than healthy lungs and therefore it seems to be much easier to observe a proinflammatory response in 'two-hit models' (5,8).

The inflammatory reaction is not confined to the lungs, but also involves the systemic circulation and affects end-organs (26). This loss of compartmentalization combined with the ventilator-induced amplification of inflammation in acute lung injury, may also be an important mechanism of MODS, one of the most common causes of death in acute respiratory distress syndrome (ARDS). In patients with ARDS, concentrations of TNF-α, IL-1ß and IL-6 were higher in the arterialised blood (obtained via wedged pulmonary artery catheter) compared with mixed venous blood, suggesting that the lungs were a major source of systemic proinflammatory cytokines in these patients (27). Thus, it was to be expected that in our study, highP not only increased MIP-2 and IL-1ß concentrations in the BAL but also in plasma.

Nitrite (NO_2^-) production was measured as a surrogate of macrophage nitric oxide production, a recognized functional marker of macrophage activation (28). Reactive nitrogen species cause tissue damage by lipid and DNA oxygenation and the inactivation of enzymes and proteins. In our model, high-pressure ventilation significantly increased the NO_2^- release from cultured alveolar macrophages. Frank *et al* (29) showed in an animal model that high tidal volume ventilation increases inducible nitric oxide synthase (iNOS) expression and air space total NO_2^-. Furthermore, Gessner *et al* (30) were able to demonstrate a direct correlation between exhaled breath condensate (EBC) NO_2^- concentration and V_T in acute lung injury. In addition, the ratio of EBC NO_2^- to V_T directly correlates to the extent of lung injury (as per the American and European Consensus Conference on the definition of ARDS).

Heat shock proteins (HSPs) are produced as a response of cells to stress such as heat, cytokines, and oxygen radicals (31). It seems reasonable that stressing lung tissue by high-pressure ventilation also induces HSPs. This was reported previously by Vreugdenhil *et al* (32) and was confirmed by our study. HSP-70 is a molecular chaperone that is involved in maintaining the conformational and structural integrity of intracellular proteins. HSP-70 expression in the lung may be regarded as a marker for pulmonary stress (33), but it is also associated with a decrease in mortality after acute lung injury (34). The mechanisms by which HSPs may be protective have not been fully elucidated, but Ribeiro *et al* (20) proposed that HSPs bind to cytokines, thus preventing their release from inflammatory cells.

Matrix metalloproteinases (MMPs) are a group of zinc enzymes responsible for the degradation of extracellular matrix components such as collagen, and they play a significant part in embryogenesis and remodelling. However, MMPs also play an important role in certain pathological processes, including lung diseases such as bronchial asthma, interstitial lung diseases and acute lung injury (35). MMP-2 is expressed constitutively by a number of cells, including endothelial and epithelial cells. MMP-9 is produced in several types of inflammatory cells, including neutrophils and alveolar macrophages (36). Cyclic mechanical stress activates alveolar macrophages *in vitro* and their release of MMP-9 (37). MMP-9 is a major factor in neutrophil transmigration, and the inhibition of MMP-9 protects against the development of VILI by the downregulation of neutrophil-mediated inflammation (38). Foda *et al* (36) found by mRNA *in situ* hybridization that high-volume ventilation caused an up-regulation of MMP-2 and MMP-9 in the lung. Furthermore, it has been shown that the number of neutrophils in the BAL correlates with an increase in MMP-9 levels and that higher concentrations of MMP-2 and MMP-9 can be found in the BALF of patients with ARDS compared to healthy volunteers (39). Similarly, Delclaux *et al* (40) demonstrated that activated MMP-2 and MMP-9 in the epithelial lining fluid were elevated in ARDS patients and that this increase correlated with increased alveolar albumin concentration. It was concluded that these gelatinases are involved in the increase in permeability of the alveolar capillary membrane characteristic of ARDS. These findings support our results showing high levels of MMP-2 and MMP-9 activity in the BAL of animals undergoing high-pressure ventilation, together with the infiltration of inflammatory cells in the lung tissue, increase in neutrophil counts in the BAL and protein leakage.

In conclusion, we were able to show that mechanical ventilation for 20 min with a p_{max} of 45 cm H_2O and PEEP of 0 not only caused severe alterations in pulmonary gas exchange and high mortality but also resulted in local and systemic inflammation, which were characterized in detail in the present study. Since lung-borne stress-induced proinflammatory mediators may play a crucial role in the development of ventilator-induced lung injury and subsequent multiple organ dysfunction syndrome, we reason that the animal model presented here has the potential to reflect the impact and connection of VILI, biotrauma and MODS and may be suitable to study the effects of therapeutic interventions on both inflammation and mortality in the near future.

Acknowledgements

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