Ventilator-Induced Mediator Release: role of PEEP and surfactant

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Ventilator-Induced Mediator Release: role of PEEP and surfactant

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Contents

Chapter 1

Lung protective ventilation in ARDS: role of mediators, PEEP and surfactant

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ABSTRACT

Lung protective ventilation such as the ARDSnet low tidal volumes strategy can reduce mortality in ARDS patients. The knowledge that an essential therapy such as mechanical ventilation on the intensive care influences patient outcome has given rise to the re-evaluation of current ventilation practices.

This review addresses the current state of lung protective strategies and their physiological rationale. Latest knowledge on the instigation and progression of lung injury by mechanical ventilation is explored, particularly the interaction between ventilation and the inflammatory response occurring in an ARDS lung. Furthermore, the role of tidal volume, PEEP, recruitment maneuvers and surfactant on lung injury is discussed. Finally, we discuss results from clinical studies on mechanical ventilation and elucidate these results with data acquired in experimental studies. Guidelines for future strategies and/or investigations are presented.

INTRODUCTION

At the dawn of the new millennium the acute respiratory distress syndrome (ARDS) network showed unequivocally that mechanical ventilation influences patient outcome [1]. They compared two ventilation strategies, the first using traditional ventilation (normal tidal volumes) and the second using reduced tidal volumes, deemed to be protective. Using the protective strategy the ARDS network reduced mortality to 31% compared to 40% in the traditionally ventilated group [1].

Thus, protective ventilation can be defined by reducing tidal volume according to the ARDS network protocol without any need for further investigation. Or has the ARDS network study started a new age of protective ventilation strategy research?

HISTORY

ARDS was mentioned in an historic article by David Ashbaugh and colleagues in 1967 [2]. They described 12 patients with severe dyspnoea, tachypnoea, cyanosis, loss of lung compliance and diffuse alveolar infiltration seen on the chest X-ray. They observed and reported several clinical and pathological similarities with neonates with respiratory distress syndrome, notably surfactant dysfunction [2]. Over 40 years before the work of Asbaugh's group, Kurt von Neergaard [3] in 1929 was the first to suggest that surface tension plays a role in lung elasticity. He showed that the pressure necessary to fill the lung with liquid was less than half the pressure needed to fill the lung with air. His explanation for this remarkable difference was based on the assumption that in each alveolus there must be a barrier between air and fluid. This barrier could reduce the size of the alveolus according to the law of Laplace [3]. From the law of Laplace, $P = 2\gamma/r$ (P = pressure in the bubble; γ = surface tension; r = radius of the bubble), it could be concluded that a reduction of the radius of a bubble needs an equal reduction in surface tension to keep the bubble stable, which can only be accomplished by a dynamic behavior of a surface tension lowering material, which is pulmonary surfactant.

Thus, when the endogenous surfactant system is impaired, independent of the cause, the rise in surface tension will result in atelectasis formation, enlargement of the functional right-to-left shunt, pulmonary edema, impaired gas exchange and subsequent hypoxemia [4]. These

patients require mechanical ventilation to decrease their work of breathing and reverse the life-threatening hypoxemia and their respiratory acidosis [5].

WHAT DOES MECHANICAL VENTILATION DO?

During mechanical ventilation either a fixed tidal volume is set (volume controlled ventilation) or a fixed pressure is set resulting in a tidal volume which is dependent on the distensibility of the lung (pressure controlled ventilation). Although it is beyond the scope of this review to go into extensive details about these two types of mechanical ventilation, we will briefly discuss the advantages and disadvantages of both of them (for a review see [5-7]). The volume which enters a lung correlates with the airway pressure, and a pressure-volume (P-V) diagram will depict this for each individual lung.

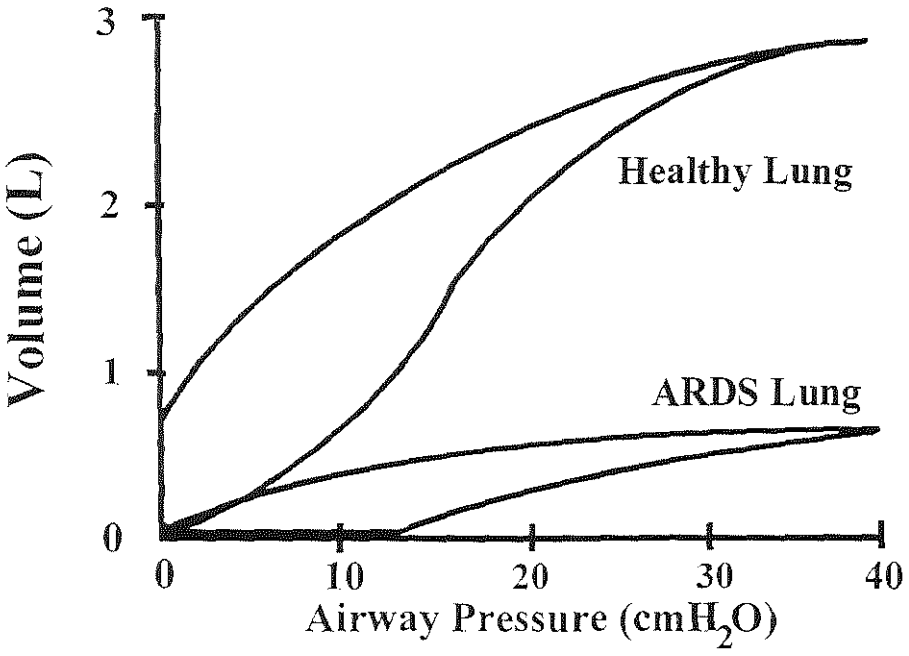

Figure 1.

Pressure volume diagram of a healthy air-filled lung and an ARDS lung. In ARDS higher pressures are required to expand the lung compared to a healthy lung due to the high surface tension at the air-liquid interface in the alveoli, which is caused by surfactant inactivity. Adapted from von Neergaard [3]

Figure 1 shows two P-V diagrams, one of a healthy lung and one of an ARDS lung (adapted from [3]). To get the same volume into an ARDS lung (which is characterised by a lower distensibility) much higher airway pressures are required compared to the healthy lung; or in other words, when applying the same airway pressure more volume will enter the healthy lung than into an ARDS lung. The P-V diagram depicted in Figure 1 demonstrates that, when using pressure controlled ventilation, airway pressures need to be adapted to the individual lung thus allowing sufficient ventilation which is a prerequisite for adequate CO_2 elimination. Setting a fixed volume will indeed allow sufficient ventilation and thus adequate CO_2 elimination, but it may lead to high airway pressures in stiff (ARDS) lungs. The high airway pressures generated in ARDS lungs are further enhanced due to the inhomogenity in distensibility of the injured lung [8], the open and thus relatively healthy lung parts will be prone to overinflation while the injured lung areas will not be inflated. The progression of the injury to the lung will result in atelectatic lung areas and patches of still open lung tissue. When this lung is ventilated, even with small tidal volumes, air will go preferentially to these open still compliant parts. This phenomenon has been described as a 'baby lung' and the subsequent ventilation even with small tidal volumes will result in overdistension [9]. Depending on the amount of collapsed lung tissue even these small tidal volumes will increase the actual tidal volume delivered to the open lung areas several fold (when 75% of the lung is collapsed, the open lung part will receive 4 times the volume in the open lung areas).

Pioneering work of Mead and colleagues demonstrated that, due to the pulmonary interdependence of the alveoli, the forces acting on the fragile lung tissue in non-uniformly expanded lungs are not only the applied transpulmonary pressures, but also the shear forces that are present in the interstitium between open and closed alveoli [10] (Fig. 2).

Transpulmonary pressures of 30 cmH_2O will result in shear forces of 140 cmH_2O [10]. Shear forces, rather than end-inspiratory overstretching, may well be the major reason for epithelial disruption and the loss of barrier function of the alveolar epithelium. In an ARDS lung there is a coexistence of collapsed alveoli, non-collapsed alveoli and alveoli that are subjected to repeated opening and closure; especially this latter category is subjected to these shear forces

[9, 10]. Important evidence for this mechanism comes from the finding that ventilation even at low lung volumes can augment lung injury in lungs with an impaired surfactant system

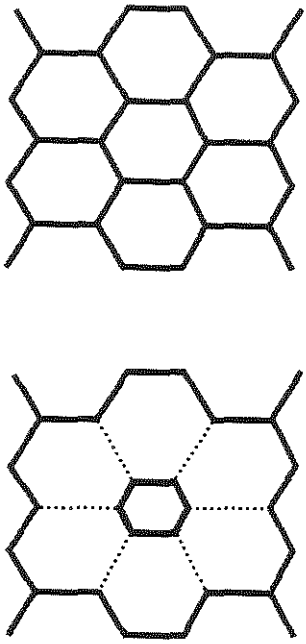

Figure 2.

Diagram showing the interdependence of alveoli. When normal alveoli are ventilated (upper panel) forces between alveoli are equal, mechanical ventilation of the same alveolar unit after surfactant inactivation (lower panel) results in end-expiratory collapse and subsequent shear forces on (adapted from [10]).

[11, 12]. Preventing repeated collapse by stabilizing lung tissue at end-expiration with positive end-expiratory pressure (PEEP) has been shown to reduce lung injury [9, 13-15].

ROLE OF PEAK PRESSURES, TIDAL VOLUME AND PEEP

Webb and Tierney in 1974 demonstrated the critical role that PEEP plays in preventing/reducing lung injury [13]. In rats ventilated with 10 cmH_2O of PEEP and a peak pressure of 45 cmH_2O no lung injury was present but using the same peak pressure and omitting PEEP severe pulmonary edema was formed within 20 min [13]. In a study by Verbrugge et al. the difference in pressure amplitude between these two groups also resulted in difference in tidal volume, i.e. 18 ml/kg and 45 ml/kg in the 45/10 and 45/0 group, respectively [14]. Dreyfuss and colleagues further explored the role of tidal volume and peak inspiratory pressures on lung injury [16]. In an animal model they applied high inspiratory

pressures in combination with high volumes which resulted in increased alveolar permeability [16]. In a second group low pressure were combined with high volume (iron lung ventilation) again resulting in alveolar permeability [16]. In the third group the effect of high pressures combined with low volume was studied, by strapping the chest wall to reduce chest excursions; the permeability of this group (high-pressure low-volume group) did not differ from the control group [16]. Thus large tidal volume ventilation increases alveolar permeability, whereas peak inspiratory pressures do not seem to influence the development of this type of lung injury. Similar observations were made in rabbits ventilated with high peak pressures in which thorax excursions were limited by a plaster cast [17]. In injured lungs the effect of higher volumes only aggravated the permeability, as demonstrated in animals in which the surfactant system was inactivated and which were subsequently ventilated with high tidal volumes [18, 19].

Although Webb and Tierney already demonstrated that PEEP could ameliorate lung injury [13], the mechanism is still not clearly understood. PEEP can stent alveoli at end expiration and thus prevent repetitive collapse, reducing shear forces [20, 21]. The most important role of PEEP is to preserve surfactant function. Two basic mechanisms have been reported to explain the surfactant-preserving effect of PEEP during mechanical ventilation. The first mechanism is alteration of the surfactant film by surface area changes, already suggested in 1972 [22]. Wyszogrodksi et al. demonstrated that PEEP could prevent collapse of the alveolar surface film due to low lung volumes in no-PEEP ventilation and thus prevent alteration of the endogenous surfactant, substantiated in this model by surface tension measurement and lung compliance [23]. Later it was shown that especially large area changes result in conversion of active surfactant (large aggregates LA) into inactive surfactant (small aggregates SA), believed to be the reason for the deterioration of surfactant function [14, 24, 25]. In the model first described by Webb and Tierney, 10 cmH_2O PEEP prevents a significant conversion of large aggregates into small aggregates compared with non-ventilated controls [14, 26]. A second mechanism explaining how PEEP preserves surfactant function, is the prevention of loss of surfactant to the proximal airways. In 1976, an ex-vivo model was used to show that ventilation caused movement of surfactant to the airways from the alveoli [27]. Preventing alveolar collapse and keeping the end-expiratory volume of alveoli at a higher level, prevents excessive loss of surfactant in the small airways by a squeeze-out mechanism during expiration [14, 15, 27, 28] (Fig. 3).

Accumulation of proteins in the lung due to influx of edema results in inactivation of surfactant [29-31]. PEEP can reduce this accumulation of protein in the lung and the subsequent inactivation of surfactant. Studying the effect of two PEEP levels Hartog and colleagues subjected rats to whole lung lavage to remove the endogenous surfactant [21]. In the first group PEEP was set to prevent hypoxemia (PEEP 8 cmH_2O) and in the other group

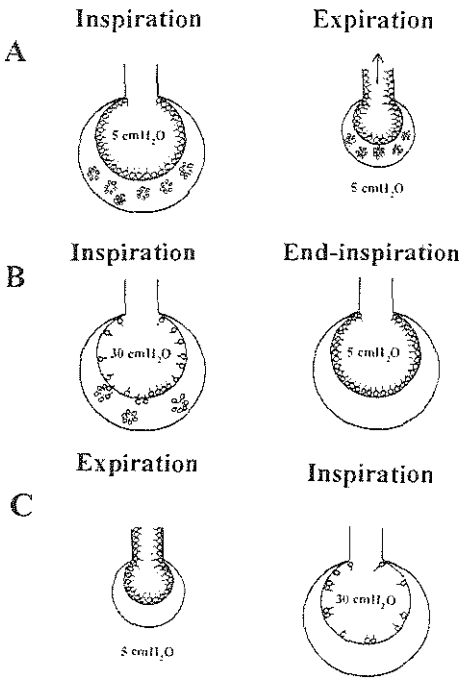

Figure 3.
Schematic representation of loss of surfactant to small airways. Balance between synthesis, release and consumption of surfactant in the healthy lung. The pressure values given represent the intrapulmonary pressure needed to open up the alveolus. A) At the surface and the hypophase (micelles), there are sufficient molecules of surfactant. These micelles deliver the surfactant necessary to replace the molecules squeezed out during expiration. B) Imbalance between synthesis, release and consumption of surfactant due to artificial ventilation. At the beginning of inspiration, there is an apparent deficiency of surfactant molecules but there is a respreading of molecules stored in the hypophase of the surfactant layer. At the end of inspiration there is, in principle, enough surfactant on the surface. C) With the next expiration, surface active molecules are squeezed out and no surface active molecules are left in the hypophase for respreading, creating the situation where a serious surfactant deficiency follows

PEEP was set to prevent collapse of alveoli (PEEP 15 cmH_2O) during the lavage procedure [21]. Although there was a similar amount of surfactant left in the lungs of both groups, there was a marked increase in alveolar protein levels in the low PEEP group, resulting in inactivation of surfactant as well as a deterioration of lung mechanics [21]. Reducing protein influx, minimizing deterioration of lung mechanics and other such protective effects by ventilating with higher levels of PEEP have been reported by others [32, 33]. Different animal models have shown that ventilation with PEEP at lower tidal volumes results in less edema than ventilation without PEEP and a higher tidal volume for the same peak or mean airway pressure [13, 16, 34, 35] and that, more specifically, PEEP prevents alveolar flooding [13, 14].

FUNCTIONS OF SURFACTANT

To allow optimal gas exchange under normobaric circumstances with minimal work effort all alveoli need to be kept open during the whole respiration period. To prevent alveoli from collapsing at the end of expiration the surface tension at the alveolo-capillary membrane needs to be reduced to keep the alveolus stable (as described earlier in the law of Laplace). Surfactant reduces this surface tension and is therefore an essential part of normal lung function.

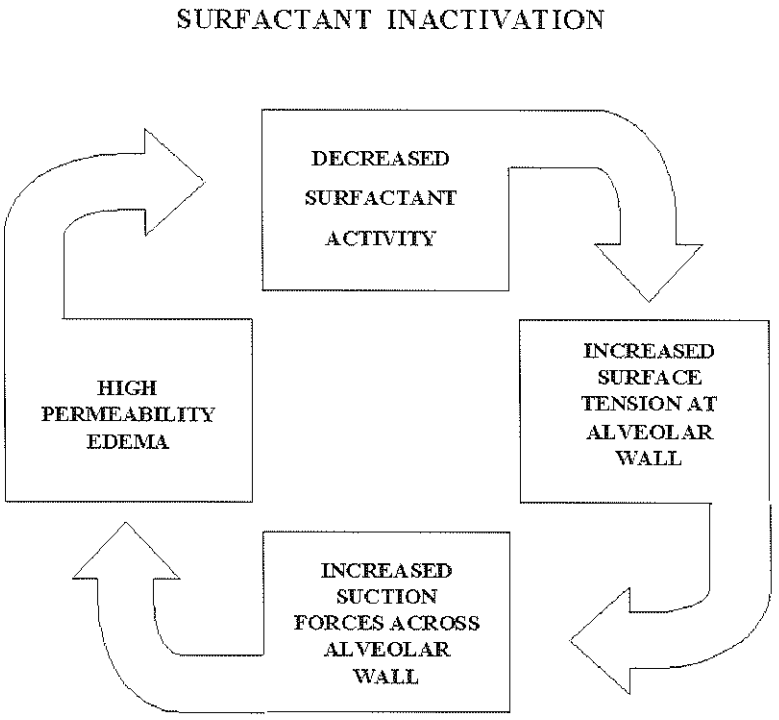

Figure 4.
Perpetuation of surfactant inactivation. Schematic pathway along which surfactant inactivation will perpetuate damage inflicted on the surfactant-alveolus system.

However, besides reducing surface tension surfactant also has several other key roles in the lung. One of the essential functions of surfactant is limiting transfer of molecules across the alveolo-capillary membrane, this has been studied with ^{99m}Tc-DTPA in models of surfactant depletion and/or inactivation [36, 37]. Pretreatment with exogenous surfactant prevents the injurious effect of large tidal volume on the clearance of ^{99m}Tc-DTPA [38]. Verbrugge et al. demonstrated that incremental doses of surfactant before ventilation with large tidal volumes and no PEEP reduced protein transfer (measured by Evans Blue) across the alveolo-capillary membrane and maintained arterial oxygenation during the 20-min observation period [39]. Due to damage of both the epithelial and endothelial barrier, surfactant components may be lost into the bloodstream.

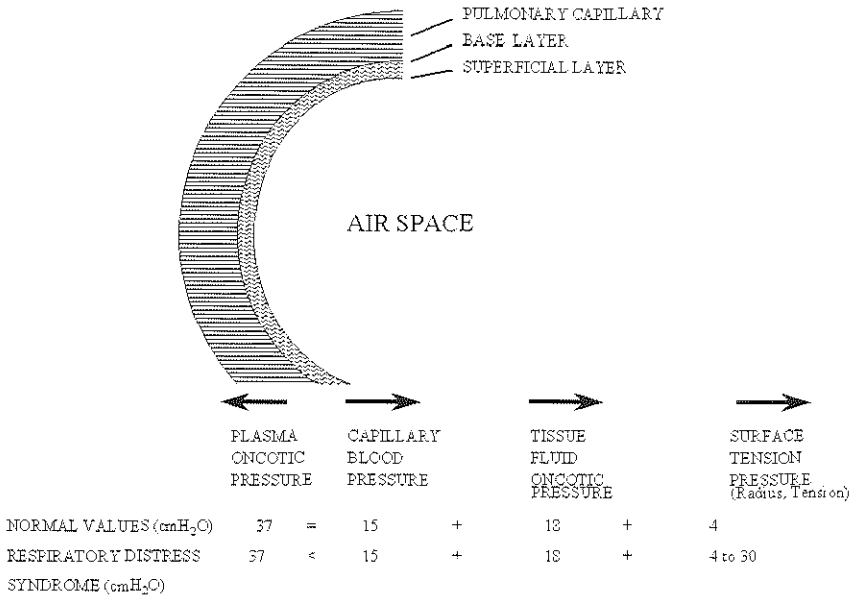

Figure 5.
Simplified diagram showing the factors influencing fluid balance in the lung (from [4]).

More importantly, protein will accumulate intra-alveolarly which results in dose-dependent inhibition of surfactant [29]. As surfactant is rate-limiting for the transfer of proteins over the alveolo-capillary barrier, loss of surfactant function will lead to further protein infiltration. This may result in a self-triggering mechanism of surfactant inactivation (Fig 4) [29-31]. The

application of PEEP has been shown to reduce protein infiltration/edema formation, as previously mentioned in several models of lung injury [14, 16, 21, 34]. This protection against lung edema is also caused by stabilization of the fluid balance in the lung, especially across the alveolo-capillary membrane. Figure 5 presents a diagram of fluid balance across the lung. The normal plasma oncotic pressure of 37 cmH_2O is opposed by the capillary hydrostatic pressure of 15 cmH_2O, the oncotic pressure of interstitial fluid proteins of 18 cmH_2O and by the surface tension conditioned suction pressure of 4 cmH_2O. In general, alveolar flooding will not occur when the surfactant system is properly functioning. However, when the surface tension rises above a critical level alveolar flooding will occur, leading to influx of proteins into the alveolar space which results in further inactivation of surfactant [29].

A second very important role of surfactant is the protection against bacteria and other micro-organisms. Surfactant (especially SP-A and SP-D) and alveolar macrophages have a synergistic effect in the defense against bacteria [40, 41]. Surfactant proteins recognize and interact with the surface of micro-organisms. They protect the lung by interacting with a wide variety of potential pathogens, including viruses, bacteria, and fungi.

Pathogens can interact directly with the extracellular surfactant pool, the release and or production during host-pathogen interaction of: proteases, phospholipases and oxygen radicals directly alter the lipids in surfactant [42, 43]. Lysis of surfactant producing type II cells by pathogens and the subsequent destruction of the integrity of the alveolar-capillary membrane will further impair replenishment of the surfactant pool [43, 44]. Studies in patients have shown that, following a decrease in lung compliance (thus, surfactant deficiency), pneumonia will often develop [45]. Restoring the amount of active surfactant in the lung will improve lung function in these patients and help restore gas exchange [46].

CYTOKINES, INFLAMMATORY MEDIATORS

In patients ventilated with a lung protective strategy (low tidal volume; high PEEP), lower levels of inflammatory mediators were found [47]; these lower levels of inflammatory mediators correlated with lower levels of multi-organ failure and thus improved patient outcome [48].

These observations that mechanical ventilation influences mediator levels and finally patient outcome are substantiated by experimental data. In a landmark article, Tremblay and colleagues demonstrated that injurious ventilation strategies could induce cytokine release [49]. Using an isolated non-perfused rat lung model they demonstrated that ventilation with high volumes (40 ml/kg bodyweight) and no-PEEP resulted in increased levels of TNF-α, IL-1β, IL-6, MIP-2, IFN-γ and IL-10; both in the presence of an inflammatory stimulus (lipopolysaccharide induced) or in a non-stimulated lung [49]. Ventilation with a lower volume (15 ml/kg bodyweight) without PEEP, resulted in only a significant effect on the above-mentioned cytokines in the pre-inflamed lung [49]. Addition of PEEP of 10 cmH_2O almost prevented this increase of cytokine release.

The observation that ventilation-induced cytokine release is dependent on the level of 'priming' of the inflammatory milieu is corroborated by other studies [50, 51]. Ricard et al. (in a similar set of experiments) failed to show any effect of ventilation on cytokine releases without a pre-stimulus [50]. Similarly Verbrugge and colleagues could not demonstrate any release of TNF-α during different ventilation strategies in vivo in healthy lungs [51].

In contrast, ventilation of an "inflamed" lung has been shown to result in release of cytokines [49, 50, 52]. One of the proposed mechanisms for increased mediator levels found in injuriously ventilated lungs or in the serum of these animals is the loss of compartmentalization [26, 53-55]. The concept of compartmentalization states that the inflammatory response remains compartmentalized in the area of the body where it is produced, i.e. in the alveolar space or in the systemic circulation [53, 55-58]. Recently, our group demonstrated that compartmentalization of TNF-α (a pro-inflammatory cytokine) is lost after ventilator-induced lung injury [26]. This loss of compartmentalization is dependent on the amount of active surfactant present at the alveolar-capillary membrane [55]. Preserving the endogenous surfactant system with PEEP will (further) reduce this loss of compartmentalization [55].

Thus, mechanical ventilation increases cytokine release in susceptible lungs. But is there a clear pathway how these cytokines are released?

ROLE OF CELLS

In ARDS the intense inflammatory process with sequential activation of cytokines, chemokines and secretion of proteases, alters the inflammatory milieu in the lung resulting in activation in the lung of several cell types [59]. Kawano et al. demonstrated that removal of neutrophils decreased ventilation-induced lung injury (superior oxygenation and less hyaline membrane formation) compared to a group in which the neutrophils were still present [60]. Alveolar macrophages have been reported to be very susceptible to mechanical stress [61]. Using a plastic lung model several lung cells were subjected to cyclic strain resembling mechanical ventilation resulting in release of several key inflammatory mediators such as TNF-α, IL-8, IL-6 and matrix metalloproteinase-9 [61]. Other cells such as endothelial cells, bronchial cells and fibroblast failed to show a response in this model [61]. However, Vlahakis et al. did show release of IL-8 in alveolar epithelial cells by stretching them during 48 hours [62]. MIP-2 (rodent homologue of IL-8) was also released by stretch in cultures of fetal rat lung cells, similar to LPS-induced release [63]. Thus, cytokine and chemokine production in the lung could be due to the shear forces generated by the inhomogenity of the lung injury observed in ARDS patients.

Studying the effect of early markers of metabolic changes in lung cells, Verbrugge et al. demonstrated that injurious ventilation resulted in release of purines into the alveolar space [64]. Purines are adenosine-triphosphate catabolites (adenosine, inosine, hypoxanthine, xanthine and urate) which are biochemical markers for ischemia [64]. Within 6 minutes after start of injurious ventilation purine levels in the lung had increased dramatically, PEEP and surfactant reduced this release of purines by preventing alveolar collapse [64]. Increased plasma levels of some purines (namely, hypoxanthine and xanthine) correlate with mortality in ARDS patients [65].

INTRACELLULAR PATHWAYS OF CYTOKINE RELEASE

Mechanical ventilation will generate pressures on lung tissue and especially on lung cells. Depending on the extent of the physical forces applied, this stress may lead to activation of pulmonary cells through mechanotransduction [66] or to rupture of membranes and tissue destruction [54].

Although it is not clear how mechanical forces are converted to biochemical signals, several pathways have been suggested such as: stretch sensitive channels, mechanoreceptors or deformation of the extracellular matrix-integrin-cytoskeleton [66, 67]. The activation of the intracellular pathways has been studied more extensively, such as the stress-activated signaling cascades of the mitogen-activated protein kinase (MAPK) dependent pathways [66, 68, 69] and activation of the transcription factor nuclear factor (NF)-κB and subsequent release of pro-inflammatory mediators [61, 69-72].

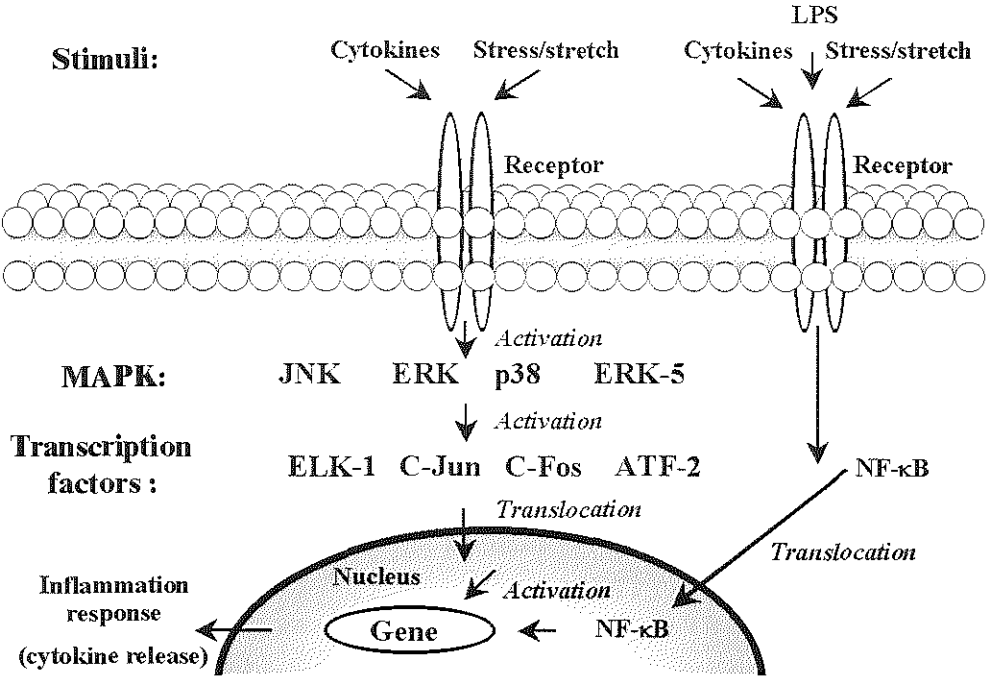

Figure 6.
Schematic representation showing how external stimuli (cytokine. stress/stretch or LPS) activate on one or more of the MAPK subfamilies (JNK, ERK, p38 and ERK-5) and/or the NF-κB pathway.
Activation of the MAPK pathway can activate transcription factors (ELK-1, C-Jun, C-Fos and ATF-2) which will subsequently be translocated to the nucleus where they can induce transcription of early response genes, resulting in an inflammation response and production of cytokines. Activation of NF-κB by external stimuli will result in translocation of this transcription factor to the nucleus and activation of gene transcription and finally in release of cytokines.

MAPK are a family of proline-targeted serine-threonine kinases that transduce environmental stimuli to the nucleus. Mammals express at least four distinctly regulated groups of MAPK:

extracellular signal-related kinases (ERK)-1/2, c-Jun amino-terminal kinases (JNK1/2/3) also know as stress-activated protein kinases, p38 kinase and ERK-5 [73]. One major function of MAPK is activation of transcription factors such as ETS like protein-1 (ELK-1), c-Jun, c-Fos, and activating transcription factor-2 (ATF-2) which control a wide variety of genes, many of which are involved in the regulation of inflammation and proliferation (Fig. 6).

NF-κB is known to be a key transcription factor for maximal expression of many cytokines that are involved in the pathogenesis of lung inflammation [66, 74]. In patients with ARDS, increased activation of NF-κB expressed by alveolar macrophages has been observed [75] and human macrophages subjected to ventilation have increased activation of NF-κB [61]. Held and co-workers demonstrated that increased activation of NF-κB can be caused either by LPS or mechanical ventilation and that this activation resulted in release of several pro-inflammatory cytokines (MIP-2, IL-6 and TNF-α) [70].

MAPK are activated by various forms of extracellular stress and might serve an important role in the cellular responses to ventilation-induced mediator release. Recently, Uhlig and colleagues demonstrated that ventilation of healthy rats with high inspiratory pressures triggered both MAPK and NF-κB pathways [69], which could contribute to the inflammation of the lung.

MECHANICAL VENTILATION CLINICAL TRIALS

In 1990 Hickling and colleagues demonstrated that mechanical ventilation could influnece mortality in ARDS patients [76]. Lowering tidal volume (TV) in a retrospective study of 50 ARDS patients decreased mortality [76]. The outcome of this study sparked renewed interest in lowering TV in ARDS patients. Three subsequent controlled trials using low TV strategies were simultaneously started but all failed to improve patient outcome [77-79]. These studies used a TV of approximately 7 ml/kg in their low tidal volume arms and a TV of 10 ml/kg in their control arms [77-79]. In contrast, using a TV of 6 ml/kg in their treatment arm and a TV of 12 ml/kg in their control arm (TV calculated by using predicted bodyweight) the ARDS network was able to reduce mortality [1]. The explanation given by the ARDS network trial for the beneficial effect on mortality was the greater difference in tidal volume between the two arms of the study, the power of the study (ARDS network studied 861 patients while the other 3 studied a maximum of 120 patients), and the aggressive treatment/prevention of

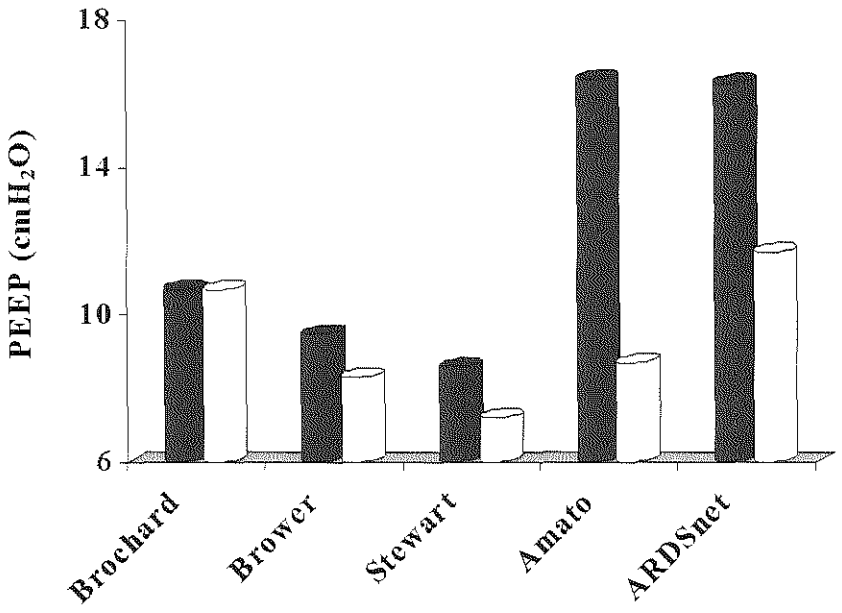

Figure 7.
Total PEEP levels applied in recent studies on protective mechanical ventilation. Studies used are by Brochard et al. [77], Brower et al. [78], Stewart et al. [79], Amato et al. [80] and the ARDSnet [1] with intrinsic PEEP modification from De Durante et al. [81, 82]. Black bars represent the PEEP levels in the lung protective strategies and the white bars the PEEP levels of the control arms of the corresponding studies.

acidosis [1]. The only other randomized controlled trial to show a reduction of mortality in ARDS patients had been published 2 years earlier. Amato et al. reported that mortality in 53 patients was significantly reduced by applying a protective ventilation strategy [80]. In their study TV was also reduced to below 6 ml/kg in the low tidal volume group compared to 12 ml/kg TV in the control arm. In contrast to the three negative studies [77-79] the PEEP level in the low tidal volume group of Amato et al. [80] was significantly higher i.e. almost 17 cmH_2O compared with 8-10 cmH_2O PEEP in the studies by Brochard et al. [77], Brower et al. [78] and Stewart et al. [79] (Fig 7). Experimental data have shown that ventilation with low tidal volumes by itself does not prevent lung injury and may even worsen lung injury when repeated collapse of lung tissue is not prevented [11]. In the ARDS network trial the low TV group had a slightly higher set PEEP of 9 cmH_2O compared to a set PEEP of 8 cmH_2O in the control group [1]. However, the increased respiratory rate (to help prevent acidosis) used in the low TV group may have resulted in

intrinsic PEEP which contributed to a higher total PEEP (16 cmH_2O) in this group [81, 82] compared to 12 cmH_2O in the traditional TV group. This higher total PEEP could help explain the decrease in mortality observed in this group (Fig. 7). Furthermore, in the ARDS network study only 12% of the screened patients were randomized while the mortality in the excluded group was higher than those included in the trial [83, 84].

PEEP levels currently employed in intensive care units around the world are below 6 cmH_2O in 78% of the patients receiving mechanical ventilation [85]. Even more disturbing is that in the same study only **three** patients of the 1638 ventilated patients studied had a PEEP level above 15 cmH_2O [85]. Whereas it is known that high PEEP levels above 15 cmH_2O are necessary to prevent repetitive collapse of alveoli and thus reduce shear forces [20]. Furthermore, only studies using PEEP levels above 15 cmH_2O in their protective arm have demonstrated a reduction in mortality [1, 47, 80, 81].

WHY DO PATIENTS WITH ARDS DIE?

Although ARDS is characterized by PaO_2/FiO_2 ratio in the American-European Consensus conference on ARDS [86], patients do not die from hypoxemia but rather die from multi-organ failure [84, 87]. Ranieri and co-workers in 2000 linked increased levels of serum inflammatory mediators to organ failure in patients suffering from ARDS [48]. These increased serum levels of inflammatory mediators were observed in patients ventilated with conventional ventilation, in contrast a lung protective ventilation strategy (high PEEP, low TV) that minimized the inflammatory response and subsequently had a lower incidence of organ failure [47, 48]. As discussed earlier, ventilation can induce mediator release especially in susceptible lungs (e.g. inflamed). Increased levels of cytokines in the serum were also observed in the ARDS network trial, in which higher levels of IL-6 were observed after 3 days of ventilation in the control arm compared to the reduced tidal volume [1]. Similarly, the number of days without non-pulmonary organ or system failure (circulatory, coagulation and renal failure) was significantly higher in the group treated with lower tidal volumes [1]. Increased levels of inflammatory mediators correlate with the development of ARDS [88] and high broncho-alveolar lavage levels of these mediators in ARDS lungs have been described extensively [89-91]. Furthermore, persistent high levels of inflammatory mediators in the lung over time correlate with poor outcome [92]. Similarly, plasma levels of

inflammatory mediators correlate with severity of ARDS and subsequently outcome [92, 93]. Headley and co-workers investigated the role of inflammatory plasma cytokines during infections and systemic inflammation and the subsequent development and progression of ARDS [93]. The final outcome of ARDS patients correlated with the magnitude and duration of the host inflammatory response in the serum and was independent of the precipitating cause of ARDS or the occurrence of infections [93]. Similar observation were made in multiple trauma patients in which high concentrations of cytokines correlated with the development of ARDS and finally multi-organ failure [94]. Our group has demonstrated that injurious mechanical ventilation can result in loss of a compartmentalized inflammation response and thus increasing serum levels of inflammatory mediators [26]; similar observations were made by Chiumello et al. [52]. Especially in the early stage of an inflammation the response will be compartmentalized, as observed in community-acquired pneumonia [58].

Because of the strong correlation between circulating inflammatory cytokines (especially TNF-α) and poor outcome during systemic inflammation, it has been suggested to decrease circulating levels of this cytokine (TNF-α) either by monoclonal antibodies or soluble receptor antagonists [95, 96]. However, in all clinical trials in which these treatments were used mortality did not decrease [95] and sometimes even increased [96]. Although none of these trials were specifically aimed at ARDS patients, they included patients with ARDS.

In healthy patients no effects on plasma levels of mediators were observed during 1 hour of mechanical ventilation; even ventilation with high tidal volumes on ZEEP did not result in higher cytokine levels compared with lung-protective ventilatory strategies [97]. Previous lung damage seems to be mandatory to cause an increase in plasma cytokines after 1 hour of high tidal volume ventilation [97].

Thus, in ARDS there is an inflamed lung with increased levels of pro-inflammatory mediators, and ventilation itself can increase the amount of inflammatory mediators produced by the lung. When the barrier function of the alveolar-capillary membrane is lost this will result in leakage of mediators to the circulation (decompartmentalization). The subsequent increased levels of these mediators in the circulation correlate with multi-organ failure and finally mortality. Use of lung protective ventilation in both experimental and clinical studies has demonstrated that a reduction in cyclic collapse of the lung which in turn reduces organ failure and mortality.

LESSONS FOR THE FUTURE

In conclusion, in an ARDS lung or a lung that is susceptible to develop ARDS a higher level of inflammation is present. When these lungs are mechanically ventilated, ventilation that will increase the inflammation response should be minimized and the barrier function of the lung should be preserved. Especially ventilation with large tidal volumes combined with end-expiratory alveolar collapse and the subsequent appearance of shear forces should be minimized. With these guidelines, circulating levels of inflammatory mediators can be reduced which can help reduce the incidence of multi-organ failure in ARDS patients and finally reduce mortality.

To minimize the effects of ventilation-induced lung injury the preferred ventilation should be pressure-controlled ventilation. When ventilating in a pressure-controlled mode the risk of overdistension of healthy parts of injured lung areas (as present in inhomogenous lung injury such as ARDS) is prevented. Use as small as possible tidal volumes in order to prevent overdistension and dangerous shear forces. Apply sufficiently high levels of PEEP to prevent end-expiratory collapse and the ensuing shear forces which will further impair lung function. Also, sufficiently high levels of PEEP can help to prevent further loss of surfactant in still healthy alveoli, halting the further spread of the disease process and reducing capillary leakage and transfer of cytokines, bacteria and other inflammatory stimuli across the alveolar capillary membrane. Finally, active recruitment of collapsed lung tissue should be considered to improve oxygenation and reduce shear forces further because ventilation at higher lung volumes reduce shear forces by reducing the coexistence of collapsed and non-collapsed alveoli [98-102]. In 1982 and 1992 Lachmann suggested such a ventilation strategy [7, 102]. Using these guidelines and applying them to the lessons already learned we can further improve the outcome of ARDS patients and reduce the effects of iatrogenic lung damage.

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Chapter 2

Ventilator-induced lung injury leads to loss of alveolar and systemic compartmentalization of TNF-α

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ABSTRACT

Objective: To determine the effect on compartmentalization of the TNF-α response in the lung and systemically after ventilation with high peak inspiratory pressure with and without PEEP.

Design: Prospective, randomized, animal study.

Setting: Experimental laboratory of a university.

Subjects: 85 male Sprague-Dawley rats.

Interventions: Lipopolysaccharide (LPS) was given intratracheally or intraperitoneally to stimulate TNF-α production, control animals received a similar amount of saline. Animals were subsequently ventilated for 20 minutes in a pressure control mode with PIP/PEEP ratio of either 45/0 or 45/10 (frequency 30 bpm, I/E ratio 1:2, FiO_2 =1).

Measurements and results: Blood gas tension and arterial pressures were recorded at 1, 10 and 20 min after start of mechanical ventilation. After sacrifice, pressure-volume curves were recorded and bronchoalveolar lavage (BAL) was performed for assessment of protein content and the small/large surfactant aggregate ratio. TNF-α was determined in serum and BAL. TNF-α levels were significantly increased after LPS stimulation, furthermore ventilation without PEEP resulted in a significant shift of TNF-α to the non-stimulated compartment as opposed to ventilation with a PEEP level of 10 cm H_2O.

Conclusions: Ventilation strategies which are known to induce ventilation–induced lung injury (VILI), disturb the compartmentalization of the early cytokines response in the lung and systemically. Furthermore, the loss of compartmentalization is a two-way disturbance, with cytokines shifting from the vascular side to the alveolar side and vice versa. A ventilation strategy (PEEP level of 10 cm H_2O) which prevents VILI significantly diminished this shift in cytokines.

INTRODUCTION

The leading cause of death in ARDS is multi organ failure (MOF) caused by SIRS (1). Recent work has suggested that detrimental modes of mechanical ventilation could contribute to MOF through the spread of inflammatory mediators (2-6). Support for this hypothesis comes from both *in vitro* and clinical studies. *In vitro*, ventilation with approximately twice the normal tidal volume (i.e. 2 x 8 ml/kg) in isolated perfused mouse lungs elicits release of inflammatory mediators into the perfusate, i.e. the systemic circulation (3, 6). Similarly, in isolated non-perfused rat lungs Tremblay and coworkers demonstrated an increase in the release of inflammatory mediators after mechanical ventilation with large tidal volume (40 ml/kg bodyweight) without positive end-expiratory pressure (PEEP) (5). In a clinical study Ranieri and colleagues showed that mechanical ventilation with large tidal volume and low levels of PEEP leads to increased levels of cytokines both in the bronchoalveolar lavage (BAL) and serum compared with patients ventilated with low tidal volumes and significantly higher levels of PEEP (2). In addition, also in the ARDSnet ventilation study, ventilation with reduced tidal volumes resulted in lower mediator levels compared with conventional ventilation (7, 8). In their study the PEEP levels used in the two groups were similar; these findings suggest that overstretching of alveolar units will result in stimulation of the immune system which may be exacerbated by the lack of PEEP.

However, in contrast to the evidence cited above, *in vivo* in uninjured lungs ventilation alone does not appear to be a sufficient stimulus for mediator release. This was shown by studies both in rats (9) and in humans (10). Altogether this suggests that ventilation-induced cytokine release in isolated organs and more importantly in ARDS patients may be explained by a two-hit model with ventilation being the second hit. One such hypothesis states that the degree of overstretching necessary to evoke cytokine release can only be achieved in isolated or inhomogeneously (pre)-injured lungs. An alternative, although not mutually exclusive explanation for the spread of mediators as a result of ventilation is loss of compartmentalization. The important concept of compartmentalization comprises the fact that the inflammatory response remains compartmentalized in the area of the body where it is produced, e.g. in the alveolar space or in the systemic circulation (11-14). Tutor *et al.* showed in isolated lungs that compartmentalization of intra-alveolar TNF-α may be lost after

chemically-induced lung injury (13). Hence the present study was undertaken to study the effect of ventilation on compartmentalization *in vivo*.

Therefore, in the present study alveolar or systemic inflammation was induced by either intratracheal or intraperitoneal injection of bacterial lipopolysaccharides (LPS) and used TNF-α as a marker to investigate the effect of different ventilation strategies on the compartmentalization of the inflammatory response.

MATERIALS AND METHODS

This study was approved by the local Animal Committee of the Erasmus University Rotterdam. Care and handling of the animals were in accordance with the European Community guidelines. The studies were performed in male Sprague-Dawley rats (n=85) with a bodyweight (BW) of 260 ±40 g (IFFA Credo, The Netherlands). An overview of the different experimental groups is presented in Table 1.

Table 1.

Overview of the different experimental groups.

Group	Instilled fluid	PIP/PEEP	n
1	5 ml/kg LPS intratracheally	45/0	10
2	5 ml/kg LPS intratracheally	45/10	10
3	15 ml/kg LPS intraperitoneally	45/0	10
4	15 ml/kg LPS intraperitoneally	45/10	10
5	5 ml/kg saline intratracheally	45/0	10
6	5 ml/kg saline intratracheally	45/10	10
7	15 ml/kg saline intraperitoneally	45/0	10
8	15 ml/kg saline intraperitoneally	45/10	10
Control	Non treated	spontaneous breathing	5

LPS: lipopolysaccharide

n; number of animals

Intratracheally treated

Two groups received lipopolysaccharide 5 ml/kg intratracheally (LPS, 1 mg/ml Salmonella Abortus Equi S form, Metalon GmbH, Wusterhausen, Germany) and another two groups received the same amount of saline (5 ml/kg) intratracheally as previously described (15). In

short, animals treated intratracheally were anesthetized with 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane; Pharmachemie BV, Haarlem, The Netherlands), and tracheotomized. A sterile metal cannula was inserted into the trachea; subsequently the operation area was infiltrated with 30 mg/kg lidocaine (xylocaine; Astra Pharmaceutical BV, Rijswijk, The Netherlands). LPS or saline was administered through the tracheal cannula over five minutes in spontaneously breathing animals; all animals recovered from anesthesia and breathed spontaneously for the ensuing 90 minutes.

Intraperitoneally treated

Animals receiving intraperitoneal administration of either LPS (15 ml/kg) or saline (15 ml/kg) were anesthetized using the same method as described above; however, no trachea cannula was inserted and consequently there was no local infiltration with lidocaine. Animals recovered from anesthesia and breathed spontaneously during the ensuing 90 minutes.

Mechanical ventilation

All animals were anesthetized again with inhalation anesthesia (see previous description) and a sterile polyethylene catheter (0.8 mm o.d.) was inserted into the carotid artery; animals which received treatment intraperitoneally were then tracheotomized and a sterile metal cannula was inserted into the trachea. After these surgical procedures, gaseous anesthesia was discontinued and anesthesia was continued with 60 mg/kg pentobarbital sodium (Nembutal; Algin BV, Maassluis, The Netherlands) given intraperitoneally during the remainder of the experiment. Muscle relaxation was attained with 2 mg/kg pancuronium bromide (Pavulon; Organon Technika, Boxtel, The Netherlands) intramuscularly. After muscle relaxation all animals were connected to a ventilator (Servo Ventilator 300; Siemens-Elema, Solna, Sweden) set in a pressure controlled mode, frequency 30 breaths/min, I/E ratio of 1:2, and a fractional inspired oxygen tension of 1.0. Depending on the ventilation group (Table 1) the animals were either ventilated with a peak inspiratory pressure (PIP) of 45 cm H_2O with zero PEEP, or PIP of 45 cm H_2O and a PEEP of 10 cm H_2O. Blood pressure was monitored through the carotid artery and at 1, 10 and 20 minutes after starting ventilation blood samples were taken to analyse blood gases using conventional methods (ABL 505; Radiometer, Copenhagen, Denmark). After 20 minutes of ventilation all animals were killed with an overdose of pentobarbital sodium.

Control group

The control group received no treatment and was not ventilated; after 150 minutes (approximate time elapsed between start of anesthesia and end of experiment in each treated animal) the trachea and carotid artery were cannulated as previously described using the same gaseous anesthesia mixture. At the end of these procedures animals were killed with an overdose of intra-arterial administered pentobarbital.

Bronchoalveolar lavage and lung mechanics

Heperanized blood samples were taken before the sacrifice of each animal. Supernatant of blood was centrifuged at 4°C at 400 *g* for 10 minutes to remove cells and cellular debris, snap-frozen on liquid nitrogen and stored at -80°C until further analysis.

After the animals were killed a static pressure-volume plot was recorded using conventional techniques (16). Maximal compliance (C_{max}) was defined as the steepest part of the pressure-volume deflation curve, and was determined separately for each animal. From the P-V deflation curve, the lung stability index was calculated according to Gruenwald (17). Thereafter BAL was performed with saline 1.5 mM $CaCl_2$ (30 ml/kg heated to 37°C) five times; percentage recovery was calculated. BAL was centrifuged at 4°C at 400 *g* for 10 minutes to remove cells and cellular debris. Supernatant of BAL was taken and snap-frozen on liquid nitrogen and stored at -80°C until further analysis.

From the BAL, the ratio of inactive/active surfactant components (SA/LA) was calculated as previously described (15). Protein concentration in the supernatant of the 40,000 *g* centrifugation was determined using a photospectrometer (Beckman DU 7400, Fullerton, CA, USA) at 595 nm applying the Bradford method (Bio-Rad protein assay, Munich, Germany) with bovine serum albumin (Sigma St Louis, MO, USA) as a standard (18).

TNF-α measurements

Rat TNF-α was assessed in blood and BAL supernatant by rat specific enzyme-linked immunosorbent assay (ELISA) obtained from Endogen (Endogen Inc., Woburn, MA, USA).

Statistical analysis

Values in the tables are expressed as mean ± SD and in the figure as mean ± SEM. Since alveolar and serum TNF-α levels in the same animal represent a paired measurement, (log-transformed) TNF-α levels were analyzed by three factorial repeated measurement ANOVA (JMP 3.01, SAS Institute, Cary, NC, USA) with treatment (LPS or saline), administration route (intratracheal or intraperitoneal) and PEEP (0 or 10 cm H_2O) as the factors. The

reported α-errors (Wilks' lambda) are from the within subjects analysis and were adjusted for multiple comparisons by the Bonferroni-Holm procedure. Inter-group comparison for all other data were analysed by one factorial ANOVA with the treatments as the factor. If ANOVA resulted in $p \leq 0.05$, a Tukey-Kramer post-test was performed. Statistical significance was accepted when $p \leq 0.05$.

RESULTS

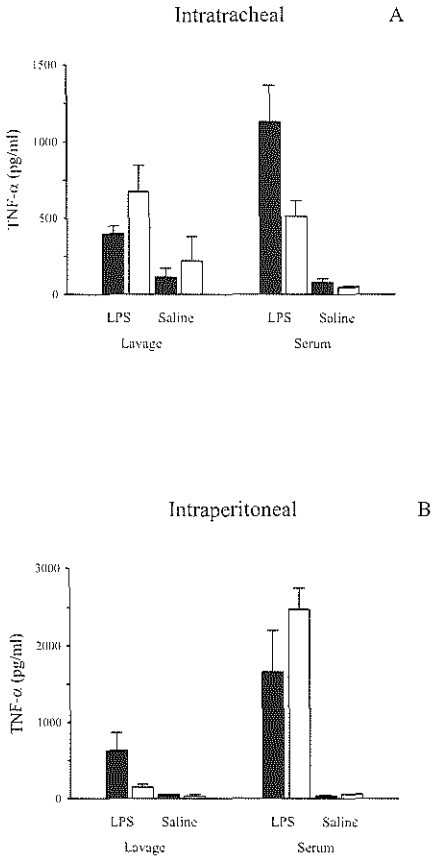

Figure 1.
Concentration of TNF-α in BAL or serum after intratracheal (A) and intraperitoneal (B) instillation of either LPS or saline. Solid bars, animals ventilated with a PIP of 45 cm H_2O and a PEEP of 0 cm H_2O; open bars, animals ventilated with a PIP of 45 cm H_2O and a PEEP of 10 cm H_2O. All data are mean ± SEM (see Results for statistical significance).

After 20 min of ventilation, TNF levels were measured in both the BAL fluid and in the serum. Figure 1 shows the TNF levels in both compartments in animals after either intratracheal (Fig. 1A) or intraperitoneal (Fig. 1B) treatment with LPS or saline. Examination of the data shows that after intratracheal challenge the intra-alveolar TNF-α levels were higher in animals ventilated with a PEEP level of 10 m H_2O compared with those ventilated with zero PEEP (Fig 1A), while in the systemic compartment it was the other way around, indicating a loss of compartmentalization of TNF-α after ventilation with zero PEEP.

Table 2.
Data on PaO_2 (mmHg), $PaCO_2$ (mmHg) mean arterial pressure (MAP)(mmHg) at 1, 10 and 20 minutes after start of mechanical ventilation in the eight ventilated groups.

	1 (LPS i.t. 0)	**2 (LPS i.t. 10)**	**3 (LPS i.p. 0)**	**4 (LPS i.p. 10)**
PaO_2 1 min	614±36	606±28	591±19	576±24
PaO_2 10 min	472±88[2,4,6]	617±29	582±58	598±12
PaO_2 20 min	387±152[2,4,6,8]	603±33[1,3,5,7]	314±187[2,4,6,8]	606±11[1,3,5,7]
$PaCO_2$ 1 min	25±4[2,4,6]	42±7[1,3,5,7,8]	26±3[2,4]	41±5[1,3,5,7]
$PaCO_2$ 10 min	20±3[2,4,6,8]	40±10[1,3,5,7]	20±3[2,4,6,8]	43±4[1,3,5,7]
$PaCO_2$ 20 min	17±3[2,4,6,8]	42±9[1,3,5,7]	22±4[2,4,6,8]	44±5[1,3,5,7]
MAP 1 min	72±13	62±22[8]	80±19	79±11
MAP 10 min	79±19	74±26	88±9	94±9
MAP 20 min	62±24	88±20	70±9	94±10
	5 (SAL i.t. 0)	**6 (SAL i.t. 10)**	**7 (SAL i.p. 0)**	**8 (SAL i.p. 10)**
PaO_2 1 min	635±28	612±31	580±13	571±28
PaO_2 10 min	590±82	626±33	572±27	567±34
PaO_2 20 min	383±135[2,4,6,8]	620±31[1,3,5,7]	265±109[2,4,6,8]	600±9[1,3,5,7]
$PaCO_2$ 1 min	20±6[2,4,6,8]	36±5[1,5,7]	24±2[2,4,6]	33±9[2,5]
$PaCO_2$ 10 min	17±6[2,4,6,8]	37±6[1,3,5,7]	19±1[2,4,6,8]	41±7[1,3,5,7]
$PaCO_2$ 20 min	19±3[2,4,6,8]	38±6[1,3,5,7]	21±1[2,4,6,8]	42±5[1,3,5,7]
MAP 1 min	59±15[8]	66±12	83±22	91±6[2,5]
MAP 10 min	92±39	69±24	94±17	81±11
MAP 20 min	81±31	66±14	80±19	91±6

Values are mean ±SD. Statistical differences have been indicated. [1] significant difference vs group 1; [2] significant difference vs group 2; [3] significant difference vs group 3; [4] significant difference vs group 4; [5] significant difference vs group 5; [6] significant difference vs group 6; [7] significant difference vs group 7; [8] significant difference vs group 8. Instilled fluid LPS (lipopolysacharide) or SAL (saline) administered either: i.t. (intratracheal) or i.p. (intraperitoneal), 0 ventilated with zero PEEP; 10 ventilated with 10 PEEP.

Similarly, Fig. 1B shows that animals injected with intraperitoneal LPS and ventilated with a PEEP level of 10 cm H_2O had no significant increase in intra-alveolar TNF-α levels. However, if intraperitoneal LPS treatment was followed by zero PEEP ventilation, this resulted in significantly higher levels of TNF-α in the alveolar space again indicated loss of compartmentalization. Concomitantly, the serum TNF-α levels were significantly lower after ventilation without PEEP compared with ventilation with PEEP (Fig. 1B).

Table 3.
Recovery of BAL fluid and post-mortem data of all nine groups for: Gruenwald index, Cmax, protein (Prot.) concentration of the BAL, total phosphorus (phosp) of small aggregates (SA) and large aggregates (LA) and the ratio of SA/LA.

	1 (LPS i.t. 0)	**2 (LPS i.t. 10)**	**3 (LPS i.p. 0)**	**4 (LPS i.p. 10)**
Recovery BAL fluid (%)	90 ± 2	88 ± 3	89 ± 3	90 ± 1
Gruenwald index	0.24 ± 0.06[2,4,6,8,9]	0.83 ± 0.21[1,3,4,5,7,8,9]	0.32 ± 0.09[2,4,6,8,9]	1.06 ± 0.02[1,2,3,5,7]
Cmax (ml/cm H_2O/kg)	2.41 ± 0.66[2,4,6,8,9]	4.52 ± 1.02[1,3,5,7]	1.94 ± 0.41[2,4,6,8,9]	4.99 ± 0.49[1,3,5,7]
Prot. conc. BAL (mg/ml)	1.00 ± 0.11[2,4,6,8,9]	0.68 ± 0.18[1,3,5,7,8,9]	1.08 ± 0.14[2,4,6,8,9]	0.47 ± 0.09[1,3,5,7]
Total phosp (SA)(mmol)	2.10 ± 0.73[4,6,8,9]	1.65 ± 0.53[4,8,9]	1.55 ± 0.29[4,6,8,9]	0.77 ± 0.30[1,2,5]
Total phosp (LA)(mmol)	0.71 ± 0.18[2]	1.20 ± 0.28[1,35,6,7,9]	0.30 ± 0.13[2,4]	0.85 ± 0.36
SA/LA ratio	3.17 ± 1.40	1.47 ± 0.64[3,5,7]	5.78 ± 2.09[2,4,6,8,9]	1.02 ± 0.62[3,5,7]

	5 (SAL i.t. 0)	**6 (SAL i.t. 10)**	**7 (SAL i.p. 0)**	**8 (SAL i.p. 10)**	**Control**
Recovery BAL fluid (%)	89 ± 2	88 ± 2	87 ± 2	91 ± 2	89 ± 3
Gruenwald index	0.33 ± 0.07[2,4,6,8,9]	0.98 ± 0.06[1,3,5,7]	0.37 ± 0.18[2,4,6,8,9]	1.08 ± 0.07[1,3,5,7]	0.99 ± 0.07[1,2,3,5,7]
Cmax (ml/cm H_2O/kg)	1.68 ± 0.13[2,4,6,8,9]	5.15 ± 0.13[1,3,5,7]	1.75 ± 0.03[2,4,6,8,9]	5.48 ± 0.48[1,3,5,7,9]	4.11 ± 0.60[1,3,5,7,8]
Prot. conc. BAL (mg/ml)	1.08 ± 0.07[2,4,6,8,9]	0.48 ± 0.20[1,3,5,7]	1.03 ± 0.09[2,4,6,8,9]	0.45 ± 0.04[1,2,3,5,7]	0.25 ± 0.02[1,2,3,5,7]
Total phosp (SA)(mmol)	2.61 ± 0.42	0.90 ± 0.53[1,5]	1.79 ± 0.57[8,9]	0.69 ± 0.55[1,2,5,7]	0.33 ± 0.17[1,2,3,5,7]
Total phosp (LA)(mmol)	0.62 ± 0.27[2]	0.71 ± 0.05[2]	0.39 ± 0.15[2]	0.71 ± 0.15[2]	0.65 ± 0.29[2]
SA/LA ratio	3.31 ± 0.81[2,4,6,8,9]	1.29 ± 0.77[3,5,7]	5.11 ± 2.77[2,4,6,8,9]	0.89 ± 0.61[3,5,7]	0.47 ± 0.12[3,5,7]

Values are mean ±SD. Statistical differences have been indicated. [1] significant difference vs group 1; [2] significant difference vs group 2; [3] significant difference vs group 3; [4] significant difference vs group 4; [5] significant difference vs group 5; [6] significant difference vs group 6; [7] significant difference vs group 7; [8] significant difference vs group 8. Instilled fluid LPS (lipopolysacharide) or SAL (saline) administered either: i.t. (intratracheal) or i.p. (intraperitoneal), 0 ventilated with zero PEEP; 10 ventilated with 10 PEEP.

To analyse these data, we examined the effects of the three factors (treatment, administration route, PEEP level) on the paired serum and lavage TNF levels (repeated measurement analysis), i.e. we investigated which of the factors affected the compartmentalization of TNF. Animals receiving LPS had a significantly higher response of TNF-α production compared with the saline groups ($p<0.0001$). Since there was a statistical interaction effect between PEEP and administration route ($p<0.0001$), these two effects had to be further analyzed by subgroup analysis (post tests): The ventilation with either 0 or 10 cm H_2O PEEP made a significant difference in (paired) TNF levels in both intraperitoneal ($p=0.0015$) and intratracheal ($p=0.0051$) treated animals, demonstrating the importance of the PEEP level for compartmentalization. Examined at a PEEP level of 10 cm H_2O, intratracheal and intraperitoneal treatment resulted in significantly different (paired) TNF levels ($p<0.0001$). However, at a PEEP level of 0 cm H_2O, there was no difference in the (paired) TNF levels in intratracheal and intraperitoneal treated animals ($p=0.9709$), showing that compartmentalization was lost In the absence of PEEP. Taken together, these data indicate that compartmentalization is maintained at a PEEP of 10 cm H_2O, but not at a PEEP of 0 cm H_2O.Table 2 gives the data on PaO_2, $PaCO_2$ mean arterial pressure (MAP) over time of the eight ventilated groups. At t=1 min PaO_2 was comparable in all groups; in the groups ventilated with zero PEEP, after 20 min PaO_2 dropped significantly, whereas in the group ventilated with a PEEP of 10 cm H_2O, PaO_2 remained stable. MAP was comparable in all groups during the whole observation period (Table 2). In the animals ventilated in a more injurious ventilatory setting (zero PEEP, PIP of 45 cm H_2O) total protein levels were significantly higher than in healthy non-ventilated control animals, whereas Cmax and Gruenwald index were significantly lower than in healthy non-ventilated control animals, indicating severe lung injury (Table 3). In the group ventilated with a PEEP level of 10 cm H_2O the Gruenwald index, Cmax and protein levels were not significantly different from the non-ventilated control animals (Table 3). The surfactant element ratio of SA/LA (e.g. inactive/active surfactant ratio) was significantly higher in the animals ventilated with a VILI mode compared with animals ventilated with a PEEP of 10 cm H_2O and with the non-ventilated control animals (Table 3).

DISCUSSION

This study demonstrates that a ventilation strategy which is known to induce VILI (19) disturbs the compartmentalization of the early cytokines response in the lung or systemically. Furthermore, the loss of compartmentalization is a two-way disturbance, with cytokines shifting from the vascular side to the alveolar side and vice versa. A ventilation strategy which prevents VILI (i.e. PEEP level of 10 cm H_2O (19)) significantly diminished this shift in cytokines.

The results of the present study show a direct link between lung injury due to VILI and leakage of cytokines across the alveolar-capillary membrane. Our data are in line with the results of Chiumello *et al.* who demonstrated that mechanical ventilation with a large tidal volume and no PEEP resulted in increased serum cytokine levels after respiratory failure due to hydrochloride acid instillation as compared to ventilation with high tidal volume and a PEEP of 5 cm H_2O (4). That group suggested that the injurious ventilatory strategy increased permeability of the alveolar-capillary membrane resulting in the loss of compartmentalization of the alveolar TNF-α response; however, interpretation of their results was confounded by the lung damage already present before a change in ventilation strategy, the presence of alveolar edema, and more importantly by the lack of data on vascular permeability such as alveolar protein levels (4). Increased alveolar protein levels are a widely accepted index of alveolar edema. Although in alveolar edema the net flow of fluid is directed inwards, the present study has provided evidence that following disruption of the alveolar-capillary border cytokines may leak from the alveolar compartment to the vascular compartment. A similar mechanism might explain the development of bacteremia after pneumonia. In line with this we have shown that mechanical ventilation without PEEP promoted bacteremia in a rat model of *Klebsiella pneumonia* (20). Rats infected with a *K. pneumonia* inoculum had negative blood cultures before the start of mechanical ventilation whereas after 3 hours of ventilation without PEEP blood cultures were positive in 70% of the animals, indicating that bacteria translocated from the lung to the vascular side of the alveolar-capillary membrane (20). The exact mechanism of this translocation is not clear, but PEEP was able to prevent this translocation. The same effect was observed in the present study: when a PEEP level of 10 cm H_2O was applied the cytokine shift was significantly reduced, preventing loss of compartmentalization. It should be noted, however, that the design of the present study does

not allow to discriminate whether it was the lack of PEEP or the higher tidal volume in the zero PEEP groups that caused the translocation of TNF-α. However, it has been shown that mechanical ventilation at low airway pressures with small tidal volumes can augment lung injury when an insufficient level of PEEP is applied (21).

The present study also demonstrates that in previously healthy lungs high levels of pro-inflammatory mediators can accumulate due to mechanical ventilation, formed systemically in response to intraperitoneal LPS, whereas the control group which received saline intraperitoneally had no significant increase in either intraalveolar or intraperitoneal TNF-α levels. We believe that cytokines, and possibly many other inflammatory mediators, will enter the lung alongside the edema liquid which enters the alveoli during lung injury (22). The shift of cytokines was already apparent after only 20 minutes of injurious mechanical ventilation, indicating that even a relatively short period of ventilation can lead to translocation of cytokines. To our knowledge, the leakage of inflammatory mediators into the lung from a systemic inflammation process has not been described before. We speculate that this could offer an explanation for the high incidence of lung failure in MOF and its susceptibility to be the initial organ to fail (23).

Although several studies have shown increased TNF levels in the BAL after mechanical ventilation with low PEEP levels, we could not demonstrate this *in vivo* (9). TNF production in our model was compartmentalized before start of ventilation, as demonstrated previously (9). In the latter study TNF was only detectable in the LPS stimulated (i.t. or i.p.) compartment respectively lung or serum in non-ventilated animals. Tutor *et al.* showed that in isolated rat lungs a challenge of LPS resulted in a compartmentalized response; however, when the lung was injured by instilling α-naphthylthiourea (ANTU) compartmentalization was lost resulting in leakage of TNF-α to the perfusate (13). While a toxic dose of TNF-α may itself lead to small pulmonary edema in rat lungs *in vivo* (24), Tutor and colleagues showed that high levels of TNF-α alone did not result in leakage of compartmentalized TNF-α from the lung to other compartments in blood-free perfused lungs (13). Similar observations were made by Ghofrani and co-workers who observed intact endothelial and epithelial barrier functions in spite of high levels of TNF-α both intravascular and alveolar (12). Although we cannot exclude an effect on the vascular permeability by the cytokines in our model, the fact that animals with the highest TNF-α serum levels (group 8) had low TNF-α levels in their lavage fluid (Fig. 1B) suggests that pulmonary compartmentalisation was not

affected by TNF-α itself. These findings are in line with data from a murine peritonitis model in which high serum cytokine levels did not increase either alveolar cytokine or protein levels (25).

In summary, this study shows that use of a mechanical ventilation mode which induces lung injury results in loss of compartmentalisation of the early cytokine response in the lung or systemically. Applying a PEEP level of 10 cm H_2O significantly diminished this loss of compartmentalisation. It was also demonstrated that this cytokine shift is not unidirectional. When translated to the clinical settings, our data advocate the use of sufficiently high levels of PEEP to prevent disruption of a compartmentalized cytokine response (in the lung or vascular) by mechanical ventilation, which otherwise might lead to MOF.

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Chapter 3

Exogenous surfactant reduces ventilator-induced decompartmentalization of TNF-α in absence of PEEP

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ABSTRACT

Objective: To determine the effect of pre-treatment with exogenous surfactant on ventilator-induced decompartmentalization of TNF-α

Design: Prospective, randomized, animal study.

Setting: Experimental laboratory of a university.

Subjects and Interventions: 102 male Sprague-Dawley rats. All animals received LPS either intratracheally or intraperitoneally to stimulate TNF-α production; half of the animals were pretreated with surfactant. Subsequently, animals were ventilated for 20 min with a peak inspiratory pressure/PEEP ratio of either 45/0 (VILI) or 45/10 (frequency 30 bpm, I/E ratio 1:2, FiO_2 =1).

Measurements and results: Blood gas tension and arterial pressures were recorded at 1, 10 and 20 min after start of mechanical ventilation. After the animals were killed, pressure-volume curves were recorded, and bronchoalveolar lavage (BAL) was performed for assessment of protein content and the small/large surfactant aggregate ratio. TNF-α was determined in serum and BAL. Pretreatment with surfactant decreased decompartmentalization of TNF-α during 45/0 ventilation. Addition of a PEEP level of 10 cm H_2O reduced decompartmentalization even further. In addition, surfactant prevented deterioration of oxygenation and decreased accumulation of protein in the BAL in the zero-PEEP group.

Conclusions: An excess of active surfactant decreases transfer of cytokines across the alveolar-capillary membrane similar to PEEP. The combination of PEEP and surfactant reduces decompartmentalization of TNF-α even further.

INTRODUCTION

Mortality of patients with acute respiratory distress syndrome (ARDS) is directly influenced by mechanical ventilation [1]. In addition, mortality of ARDS patients correlates not only with ventilation strategies, but also with the local and systemic levels of inflammatory mediators [1-4]. The ventilation strategies found to be associated with cytokine release were ventilation with high tidal volumes and/or low levels of PEEP [1-4]. One of the proposed mechanisms for increased mediator levels in injuriously ventilated lungs is the loss of compartmentalization [5-7]. The important concept of compartmentalization comprises the fact that the inflammatory response remains compartmentalized in the area of the body where it is produced, i.e. in the alveolar space or in the systemic circulation [5, 8-10]. Recently, we have demonstrated that compartmentalization of TNF-α (a pro-inflammatory cytokine) is lost after ventilator-induced lung injury (VILI) [6].

During VILI, alterations of the endogenous surfactant system have been reported [11, 12] similar to alterations observed in ARDS patients [13]. Surfactant dysfunction increases alveolar permeability [14] and supplementation with active exogenous surfactant helps to restore the alveolar-capillary membrane function and decrease permeability [15].

Therefore, we speculated that pretreatment with exogenous surfactant to increase the amount of active surfactant at the alveolar-capillary membrane, could reduce the loss of compartmentalization of TNF-α due to VILI.

MATERIALS AND METHODS

This study was approved by the Animal Committee of the Erasmus University Rotterdam. Care and handling of the animals were in accordance with the European Community guidelines. The studies were performed in male Sprague-Dawley rats (n=102) with a bodyweight (BW) of 260 ±40 g (IFFA Credo, The Netherlands). An overview of the different experimental groups is presented in Table 1.

Surfactant pretreated animals

Animals pretreated with surfactant were anesthetized with 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane; Pharmachemie BV, Haarlem, The Netherlands), and tracheotomized. A sterile metal cannula was inserted into the trachea; subsequently the

operation area was infiltrated with 30 mg/kg lidocaine (xylocaine; Astra Pharmaceutical BV, Rijswijk, The Netherlands). Exogenous natural surfactant (HL-10, Leo Pharmaceuticals Products, Ballerup, Denmark) 400 mg/kg bodyweight (dissolved in 50 mg/ml of saline) was administered through the tracheal cannula over five minutes in the spontaneous breathing animals; all animals recovered from anesthesia and breathed spontaneously for the ensuing 60 minutes to allow the instilled surfactant to be adsorbed.

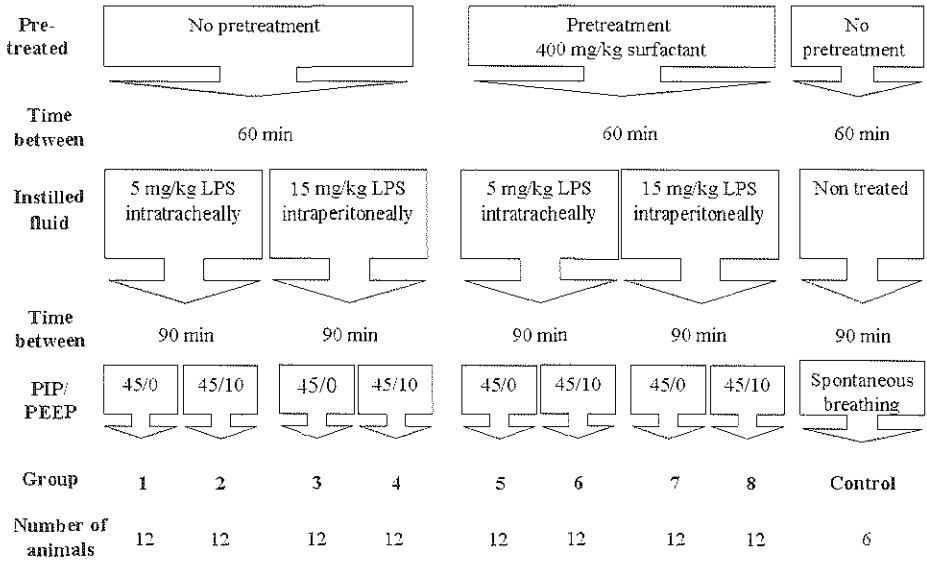

Table 1.
Overview of the nine experimental groups.

Intratracheally administered

The groups received lipopolysaccharide (LPS) 5 ml/kg intratracheally (1 mg/ml Salmonella Abortus Equi S form, Metalon GmbH, Wusterhausen, Germany) as previously described [16]. In short, animals treated intratracheally were (re)anesthetized as described above, if they were pretreated with surfactant the operation area was infiltrated again with 30 mg/kg lidocaine. In the animals that were not pretreated with surfactant the tracheal cannula was now placed and the operation area infiltrated with lidocaine. LPS was administered through the tracheal cannula over five minutes in spontaneously breathing animals; all animals recovered from anesthesia and breathed spontaneously for the ensuing 90 minutes.

Intraperitoneally administered

Animals receiving intraperitoneal administration of LPS (15 ml/kg) were (re)anesthetized using the same method as described above. No trachea cannula was inserted in the two groups that were not pretreated with exogenous surfactant and consequently there was no local infiltration with lidocaine. Animals recovered from anesthesia and breathed spontaneously during the ensuing 90 minutes.

Mechanical ventilation

All animals were anesthetized again with inhalational anesthesia (see previous description) and a sterile polyethylene catheter (0.8 mm o.d.) was inserted into the carotid artery; the two groups which received LPS intraperitoneally but were not pretreated with surfactant were then tracheotomized and a sterile metal cannula was inserted into the trachea. After these surgical procedures, gaseous anesthesia was discontinued and anesthesia was continued with 60 mg/kg pentobarbital sodium (Nembutal; Algin BV, Maassluis, The Netherlands) given intraperitoneally during the remainder of the experiment. Muscle relaxation was attained with 2 mg/kg pancuronium bromide (Pavulon; Organon Technika, Boxtel, The Netherlands) intramuscularly. After muscle relaxation all animals were connected to a ventilator (Servo Ventilator 300; Siemens-Elema, Solna, Sweden) set in a pressure controlled mode frequency 25 breaths/min, I/E ratio of 1:2, and a fractional inspired oxygen tension (FiO_2) of 1.0. Depending on the ventilation group (Table 1) the animals were either ventilated with a peak inspiratory pressure (PIP) of 45 cm H_2O with zero PEEP, or PIP of 45 cm H_2O and a PEEP of 10 cm H_2O, resulting in tidal volumes of approximately 48 ml/kg and 18 ml/kg, respectively [11]. Dead space was adapted to maintain $PaCO_2$ levels above 20 mmHg at t=2 min. Blood pressure was monitored through the carotid artery and at 1, 10 and 20 minutes after starting ventilation blood samples were taken to analyse blood gases using conventional methods (ABL 505; Radiometer, Copenhagen, Denmark). After 20 minutes of ventilation all animals were killed with an overdose of pentobarbital sodium.

Control group

The control group received no treatment and was not ventilated; the trachea and carotid artery were cannulated as previously described using the same gaseous anesthesia mixture, anesthesia was continued with intraperitoneal pentobarbital as described previously. After 150 minutes (approximate time elapsed between start of anesthesia and end of experiment in

each treated animal) animals were killed with an overdose of intra-arterial administered pentobarbital sodium.

Bronchoalveolar lavage and lung mechanics

Heperanized blood samples were taken before each animal was killed. Supernatant of blood was centrifuged at 4°C at 400 *g* for 10 minutes to remove cells and cellular debris, snap-frozen on liquid nitrogen and stored at -80°C until further analysis.

After the animals were killed, thorax and diaphragm were opened (to eliminate the influence of chest wall compliance and intra-abdominal pressure) and a static pressure-volume plot from the lung was recorded using conventional techniques [17]. Maximal compliance (C_{max}) was defined as the steepest part of the pressure-volume deflation curve, and was determined separately for each animal. Thereafter BAL was performed with saline 1.5 mM $CaCl_2$ (30 ml/kg heated to 37°C) five times; percentage recovery was calculated. BAL was centrifuged at 4°C at 400 *g* for 10 minutes to remove cells and cellular debris. Supernatant of BAL was taken and snap-frozen on liquid nitrogen and stored at -80°C until further analysis.

From the BAL, the ratio of inactive/active surfactant components (SA/LA) was calculated as previously described [18]. Protein concentration in the supernatant of the 40,000 *g* centrifugation was determined using a photospectrometer (Beckman DU 7400, Fullerton, CA, USA) at 595 nm applying the Bradford method (Bio-Rad protein assay, Munich, Germany) with bovine serum albumin (Sigma St Louis, MO, USA) as a standard [19].

TNF-α measurements

Rat TNF-α was assessed in blood and BAL supernatant by rat specific enzyme-linked immunosorbent assay (ELISA) obtained from Endogen (Endogen Inc., Woburn, MA, USA).

Statistical analysis

Values in the tables and figure are expressed as mean ± SD. All TNF-α data were log-transformed prior to analysis. To test the global hypothesis that surfactant and PEEP affect the decompartmentalization of LPS-induced TNF-α release, data were analyzed by two factorial repeated (alveolar and serum TNF-α levels in the same animal represent a paired measurement) measurement analysis of variance (ANOVA; JMP 4.05, SAS Institute, Cary, NC, USA) with treatment (surfactant, yes or no) and PEEP (0 or 10 cm H_2O) as the factors. If the global hypothesis was significant ($p \leq 0.05$), individual comparisons were performed by paired t-tests and the α-error was corrected according to the Bonferroni-Holm procedure.

Inter-group comparison for all other data were analysed by one factorial ANOVA with the treatments as the factor. If ANOVA resulted in $p \leq 0.05$, a Tukey-Kramer post-test was performed. Statistical significance was accepted when $p \leq 0.05$.

RESULTS

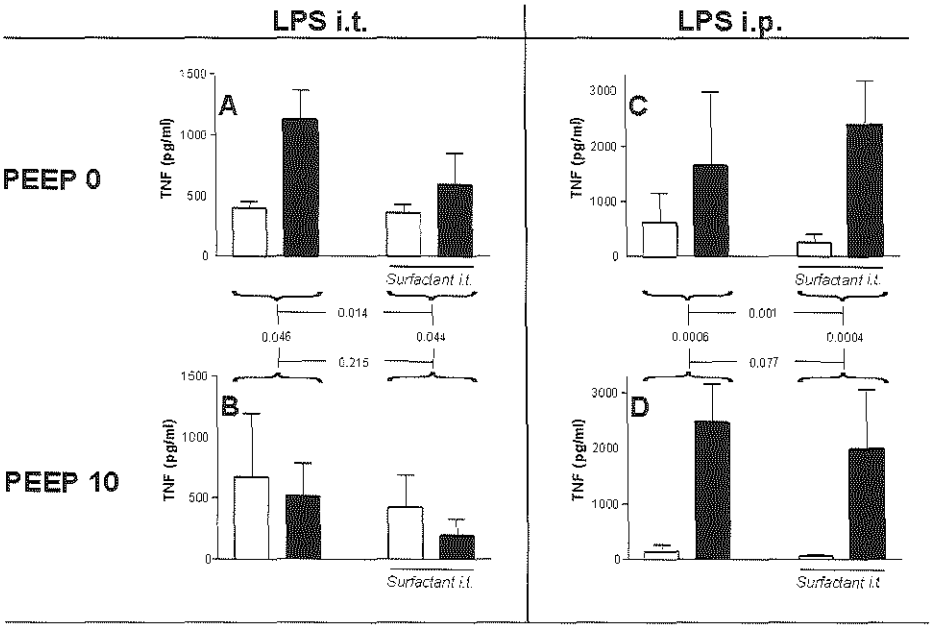

Figure 1.
Concentrations of TNF-α in BAL fluid (open bars) and in serum (solid bars). Animals were treated with LPS either intratracheally (i.t., **A** and **C**) or intraperitoneally (i.p., **B** and **D**) and ventilated with 45 cm H_2O peak-inspiratory pressure and with positive end-inspiratory pressure (PEEP) of either 0 cm H_2O (**A** and **B**) or 10 cm H_2O (**C** and **D**). On each panel (**A** - **D**) untreated animals are shown on the left side and surfactant pretreated animals on the right side.

After 20 min of ventilation, TNF levels were measured in both the alveolar lavage fluid and in the serum. Figure 1 shows the TNF levels in both compartments in animals after either intratracheal (Fig. 1 left panel) or intraperitoneal (Fig. 1 right panel) treatment with LPS only, or with surfactant and LPS.

Table 2.
Data on PaO_2 (mmHg), $PaCO_2$ (mmHg) mean arterial pressure (MAP)(mmHg) at 1, 10 and 20 minutes after start of mechanical ventilation in the eight ventilated groups.

	1(no, i.t. 0)	**2 (no, i.t. 10)**	**3 (no, i.p. 0)**	**4 (no, i.p. 10)**
PaO_2 1 min	613±34	607±26	593±19	578±22
PaO_2 10 min	471±86[2,4,5,6,7,8]	618±28[1]	581±56	599±11[1]
PaO_2 20 min	385±151[2,4,5,6,7,8]	604±31[1,3]	310±184[2,4,5,6,7,8]	606±12[1,3]
$PaCO_2$ 1 min	24±4[2,4,6,8]	42±7[1,3,5,6,7,8]	25±3[2,4]	41±5[1,3,5,6,7,8]
$PaCO_2$ 10 min	21±2[2,4,6,8]	40±10[1,3,5,7]	20±3[2,4,6,8]	44±4[1,3,5,6,7]
$PaCO_2$ 20 min	17±3[2,4,6,8]	41±9[1,3,5,7]	21±3[2,4]	44±5[1,3,5,7]
MAP 1 min	73±14[6]	63±19	81±19[6]	79±11[6]
MAP 10 min	81±18	75±26	89±8	94±8
MAP 20 min	63±24[8]	89±21	70±9	93±9

	5 (Surf, i.t. 0)	**6 (Surf, i.t. 10)**	**7 (Surf, i.p. 0)**	**8 (Surf, i.p. 10)**
PaO_2 1 min	609±56	630±42	604±46	597±38
PaO_2 10 min	573±92[1]	646±45[1]	587±73[1]	604±50[1]
PaO_2 20 min	559±108[1,3]	677±33[1,3]	565±129[1,3]	636±36[1,3]
$PaCO_2$ 1 min	25±3[2,4,6,8]	33±4[1,2,4,5,7]	24±4[2,4,6,8]	33±4[1,2,4,5,7]
$PaCO_2$ 10 min	26±3[2,4,8]	32±5[1,3,4]	27±5[2,4]	34±7[1,3,5]
$PaCO_2$ 20 min	25±4[2,4]	33±7[1]	25±7[2,4]	35±7[1]
MAP 1 min	61±17	45±10[1,3,4,7]	73±16[6]	59±18
MAP 10 min	65±15	78±32	78±26	91±20
MAP 20 min	64±23[8]	82±26	62±25[8]	98±21[1,5,7]

Statistical differences have been indicated. [1] significant difference vs group 1, [2] significant difference vs group 2, [3] significant difference vs group 3, [4] significant difference vs group 4, [5] significant difference vs group 5, [6] significant difference vs group 6, [7] significant difference vs group 7 and [8] significant difference vs group 8. No received no pre-treatment; Surf received surfactant as pretreatment; LPS (lipopolysacharide) administered either: i.t. (intratracheal) or i.p. (intraperitoneal), 0 ventilated with zero PEEP; 10 ventilated with 10 PEEP.

Table 3
Recovery of broncho-alveolar lavage (BAL) fluid and post-mortem data of all nine groups for: Gruenwald index, Cmax, protein concentration of the BAL, total phosphorus of small aggregates (SA) and large aggregates (LA) and the ratio of SA/LA.

	1(no, i.t. 0)	2 (no, i.t. 10)	3 (no, i.p. 0)	4 (no, i.p. 10)
Recovery BAL fluid (%)	91 ± 2	89 ± 3	89 ± 2	91 ± 1
Gruenwald index	0.24 ± 0.06[2,4,6,8,9]	0.83 ± 0.21[1,3,5,7]	0.32 ± 0.09[2,4,6,8,9]	1.06 ± 0.02[1,3,5,7]
Cmax (ml/cm H_2O/kg)	2.42 ± 0.67[2,4,6,8,9]	4.51 ± 1.01[1,3,5,7,8]	1.93 ± 0.42[2,4,6,8,9]	4.98 ± 0.49[1,3,5,7]
Prot conc. BAL (mg/ml)	2.00 ± 0.21[2,4,5,6,7,8,9]	1.35 ± 0.37[1,3,4,5,6,7,8,9]	2.17 ± 0.29[2,4,5,6,7,8,9]	0.95 ± 0.18[1,2,3,6,8,9]
Total phosp (SA)(mmol)	2.10 ± 0.72[3,4,5,6,7,8,9]	1.64 ± 0.51[4,5,6,7,8,9]	1.55 ± 0.29[1,4,5,6,8,9]	0.77 ± 0.31[1,2,3]
Total phosp (LA)(mmol)	0.71 ± 0.17[6,8]	1.20 ± 0.27[3,6,8]	0.30 ± 0.13[2,6,8]	0.85 ± 0.37[6,8]
SA/LA ratio	3.17 ± 1.40[2,3,4,5,6,8,9]	1.47 ± 0.64[1,3]	5.78 ± 2.09[1,2,4,5,6,7,8,9]	1.02 ± 0.62[1,3]

	5 (Surf, i.t. 0)	6 (Surf, i.t. 10)	7 (Surf, i.p. 0)	8 (Surf, i.p. 10)	Control
Recovery BAL fluid (%)	91 ± 2	89 ± 1	90 ± 2	91 ± 1	88 ± 2
Gruenwald index	0.45 ± 0.06[2,4,6,8,9]	0.81 ± 0.22[1,3,5,7]	0.51 ± 0.14[2,4,6,8,9]	0.98 ± 0.05[1,3,5,7]	0.99 ± 0.07[1,3,5,7]
Cmax (ml/cm H_2O/kg)	1.72 ± 0.38[2,4,6,8,9]	5.07 ±1.06[1,3,5,7]	1.98 ± 0.47[2,4,6,8,9]	5.93 ± 0.83[1,2,3,5,7,9]	4.11 ± 0.60[1,3,5,7,8]
Prot conc. BAL (mg/ml)	1.05 ± 0.11[1,2,3,6,8,9]	0.42 ± 0.15[1,2,3,4,5,7,8]	1.02 ± 0.13[1,2,3,6,8,9]	0.15 ± 0.08[1,2,3,4,5,6,7,9]	0.51 ± 0.03[1,2,3,4,5,]
Total phosp (SA)(mmol)	0.95 ± 0.27[1,2,3,8,9]	0.48 ± 0.15[1,2,3,7]	1.06 ± 0.30[1,2,6,8,9]	0.41 ± 0.15[1,2,3,5,7]	0.33 ± 0.17[1,2,3,5,7]
Total phosp (LA)(mmol)	0.82 ± 0.42[6,8]	1.82 ± 0.40[1,2,3,4,5,7,9]	0.69 ± 0.33[6,8]	1.99 ± 0.54[1,2,3,4,5,7,9]	0.65 ± 0.29[6,8]
SA/LA ratio	1.39 ± 0.55[1,3]	0.27 ± 0.08[1,3,7]	1.85 ± 0.86[3,6,8,9]	0.21 ± 0.06[1,3,7]	0.47 ± 0.12[1,3,7]

Statistical differences have been indicated. [1] significant difference vs group 1, [2] significant difference vs group 2, [3] significant difference vs group 3, [4] significant difference vs group 4, [5] significant difference vs group 5, [6] significant difference vs group 6, [7] significant difference vs group 7, [8] significant difference vs group 8 and [9] significant difference vs group control. No received no pre-treatment; Surf received surfactant as pretreatment; LPS (lipopolysacharide) administered either: i.t. (intratracheal) or i.p. (intraperitoneal), 0 ventilated with zero PEEP; 10 ventilated with 10 PEEP.

Intratracheal LPS administration

After i.t. challenge with LPS only, compartmentalization was lost during VILI (no PEEP), as shown by the high levels of TNF-α in the adjacent compartment (serum) (Fig. 1A, left part). In the two-way repeated measurement ANOVA, the effects of both surfactant treatment ($p=0.0084$) and PEEP ($p=0.0007$) were highly significant. Comparisons of individual groups showed that the decompartmentalization was reduced by applying a PEEP level of 10 cm H_2O ($p=0.046$, left part of Fig. 1A vs left part of Fig. 1B).

When the animals were pretreated with surfactant, the flux to the adjacent compartment (serum) was significantly reduced in the VILI group ($p=0.014$, left part vs right part of Fig. 1A). In the group ventilated with 10 cm H_2O, pretreatment with surfactant did not significantly ($p=0.215$, Fig. 1B) influence the shift of TNF-α to the serum side.

Intraperitoneal LPS administration

In the two-way repeated measurement ANOVA, the effects of both surfactant treatment ($p=0.0001$) and PEEP ($p<0.0001$) were highly significant. Comparison of the individual groups injected with intraperitoneal LPS showed that alveolar TNF-α levels were higher in animals ventilated with zero PEEP (Fig. 1C) than with 10 cm H_2O PEEP (Fig. 1D, $p=0.0006$), indicating loss of compartmentalization. When animals were pretreated with surfactant again there was a reduction in decompartmentalization in the animals ventilated without PEEP ($p=0.001$, Fig. 1C). In the animals ventilated with a PEEP level of 10 cm H_2O (Fig. 1D) pretreatment with surfactant did not affect the ratio of serum to lavage TNF concentrations ($p=0.077$).

Finally, although surfactant pretreatment reduced decompartmentalization compared to the non-surfactant treated groups, addition of PEEP at a level of 10 cm H_2O still reduced the decompartmentalization between the surfactant pretreated groups significantly (i.t. and i.p., $p=0.044$ and 0.0004, respectively).

Table 2 gives the data on PaO_2, $PaCO_2$ mean arterial pressure (MAP) over time of the eight ventilated groups. At t=1 min PaO_2 was comparable in all groups; in the groups that received no pretreatment with surfactant and were ventilated with zero PEEP, after 20 min PaO_2 dropped significantly, whereas in the groups ventilated with a PEEP of 10 cm H_2O, PaO_2 remained stable. Pretreatment with surfactant (groups 5 and 7) in the zero PEEP groups prevented deterioration of PaO_2 levels after 20 min. MAP was comparable in all groups during the whole observation period (Table 2).

In the animals ventilated in a VILI mode (zero PEEP, PIP of 45 cm H_2O) and not pretreated with surfactant, total protein levels were significantly higher than in all other groups (non-VILI groups, healthy control group and the surfactant pretreated groups) (Table 3). Ventilation with a PEEP level of 10 cm H_2O or giving surfactant before and then starting ventilation without PEEP resulted in similar protein levels which, however, were still higher than in healthy controls. Combining surfactant and a PEEP of 10 cmH_2O resulted in protein levels similar to those as observed in healthy control animals (Table 3).

In the animals ventilated with zero PEEP, Cmax and Gruenwald index were significantly lower than in healthy non-ventilated control animals. In the group ventilated with a PEEP level of 10 cm H_2O the Gruenwald index and Cmax were not significantly different from the non-ventilated control animals (Table 3). Pretreatment with surfactant did not prevent a significantly lower Cmax and Gruenwald index in the animals ventilated with zero PEEP, compared to the non-ventilated control animals and the animals ventilated with PEEP.

Ventilation with PEEP without surfactant pretreatment preserved the surfactant system (groups 2 and 4) to control values. Ventilation without PEEP and without exogenous surfactant (groups 1 and 3) resulted in a significant increase of the SA/LA aggregate ratio, indicating severely impaired surfactant function. Pretreating with surfactant while ventilating without PEEP resulted in a 'normalized' SA/LA ratio similar to the control group. Combining pretreatment with surfactant and PEEP reduced the SA/LA ratio to even below those of healthy animals (not significant)(Table 3).

DISCUSSION

This study demonstrates that increasing the amount of active surfactant at the alveolar-capillary membrane reduces ventilator-induced decompartmentalization of TNF-α. At the alveolar-capillary interface a complex balance exists between retractive and stabilizing forces (for review see [20]). Surfactant stabilizes the alveolar capillary membrane and has a rate-limiting function on the transfer of protein and other molecules across the membrane. Surfactant dysfunction leads to a net influx of fluids and proteins into the alveolus [21], as also evidenced in the present study by the increased protein levels in the lungs of animals ventilated without PEEP and without exogenous surfactant. In the present study, during VILI the surfactant system was severely impaired as demonstrated by the increased SA/LA ratio

and the deterioration in arterial oxygenation lung mechanics. These alterations in surfactant activity during VILI have been described extensively [11, 12].

Increasing the amount of active surfactant in the alveolus although it could not reduce the deterioration of lung mechanics prevented deterioration in arterial oxygenation and improved the SA/LA ratio, but most importantly it reduced the decompartmentalization of TNF-α during VILI. Surfactant protected not only against the influx of edema proteins and TNF-α (Fig. 1B), but also against the transfer of alveolar TNF-α to the serum (Fig. 1A). A similar mechanism might explain the development of bacteremia after pneumonia that developed during mechanical ventilation without PEEP in a rat model of *Klebsiella pneumonia* [22]. The exact mechanism of this translocation is not clear, but it was prevented by PEEP, which in the present study preserved the endogenous surfactant pool (SA/LA ratio). Therefore, an inverse relationship between PEEP and endogenous surfactant might explain the decompartmentalization of bacteria and mediators during VILI.

However, by studying only one cytokine (TNF-α) we are unable to state that surfactant may reduce the process of systemic inflammation. We studied the effect of exogenous surfactant therapy on the decompartmentalization of TNF-α only in order to elucidate the critical role of surfactant in the transfer of cytokines across the alveolar-capillary membrane during ventilator-induced lung injury. We chose this particular cytokine (TNF-α), because it is specifically upregulated by our inflammatory stimulus (LPS), and TNF-α has been shown to be decompartmentalized by VILI [6, 16]. Future studies should investigate whether the reduction in decompartmentalization of TNF-α also results in decompartmentalization of other inflammatory mediators and if this will cause any biological effect (e.g. multi-organ failure, higher mortality).

Verbrugge et al. demonstrated that incremental doses of surfactant before VILI reduced protein transfer (measured by Evans Blue) across the alveolar-capillary membrane and maintained arterial oxygenation during the 20 min observation period [18]. The essential function of surfactant in limiting transfer across the alveolar-capillary membrane has been studied with ^{99m}Tc-DTPA in models of surfactant depletion and/or inactivation [14, 23]. Pretreatment with exogenous surfactant prevented the injurious effect of large tidal volume on the clearance of ^{99m}Tc-DTPA [15].

Several studies have shown that ventilation alone is not sufficient to induce release of TNF-α in a healthy lung [16, 24]. Therefore, in the present study, we only compared the effect of

VILI and surfactant pretreatment on TNF-α after a 'second hit' with LPS. Although the large tidal volumes resulted in a significant lowering of arterial CO_2 levels in the 45/0 animals we did not adjust minute ventilation to prevent unwanted differences in the induced alveolar stretch per minute between the groups.

It has been reported that exogenous surfactant can modulate the TNF-α release in response to LPS [25], a phenomenon which has been ascribed to SP-A [26]. Therefore, it should be noted that in the present study we used a natural surfactant containing the surfactant proteins B and C, but not SP-A. However, we cannot completely exclude that surfactant reduced the TNF-α response to LPS by directly interfering with the activation of leukocytes. Nevertheless, the observation that the absolute intra-alveolar TNF concentrations in response to LPS instillation were not changed by surfactant (see Fig. 1A and 1B, p=0.223), argue against a direct immunomodulating effect of our surfactant preparation. In addition, in the intraperitoneally exposed animals, surfactant did not influence the TNF-α response. Therefore, the reduction measured in decompartmentalization of TNF-α in the intraperitoneal treated group is most likely dependent on the increased amount of active surfactant at the alveolar-capillary membrane.

Although surfactant reduced the ventilator-induced decompartmentalization of TNF, this was further reduced by application of PEEP. Thus, surfactant and PEEP appear to additively attenuate ventilator-induced decompartmentalization. Increasing and preserving the amount of active surfactant at the alveolar-capillary membrane may directly affect systemic cytokine levels. We therefore speculate that strategies directed to preserve endogenous surfactant (sufficient level of PEEP [6, 11]) and to increase surfactant (exogenous surfactant therapy) may beneficially influence pro-inflammatory mediator levels in patients at risk for ARDS and thus could reduce mortality in these patients. It should, however, be noted that decompartmentalization is only one of several mechanisms by which ventilation may promote cytokine release [7]. For instance, under certain conditions ventilation itself may be a stimulus for cytokine release as strong as LPS [27], although in our model this does not appear to be the case for TNF [6, 16].

In conclusion, this study indicates that surfactant dysfunction induced by VILI plays a key role in the decompartmentalization of TNF-α. Ventilator-induced decompartmentalization is best reduced by increasing the active surfactant pool in combination with PEEP.

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Chapter 4

Injurious ventilation strategies cause systemic release of IL-6 and MIP-2 in rats *in vivo*

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Submitted

SUMMARY

In vivo experiments showed no increased production of TNF in response to injurious ventilation strategies in otherwise untreated animals. Because IL-6 and MIP-2 are more sensitive markers of ventilation-induced cytokine release, serum and bronchoalveolar lavage (BAL) samples were examined for these mediators.

Adult rats were left untreated or ventilated with normal pressures (13/3; peak inspiratory pressure/PEEP in cmH_2O), with high volume and low PEEP (32/6) or high volume and no-PEEP (32/0) for 90 or 240 min. Serum and BAL samples were analyzed for IL-6 and MIP-2.

Ventilation with 32/0 for 90 or 240 min, led to increased serum IL-6 levels. Serum MIP-2 levels were increased by ventilation with 32/6 (90 min) and 32/0 (240 min). Ventilation under any condition, even at 13/3, resulted in elevated MIP-2 levels in the BAL fluid.

Even at normal pressures pulmonary MIP-2 levels were increased, suggesting that ventilation may promote pro-inflammatory responses in healthy subjects.

INTRODUCTION

Both experimental (1-3) and clinical (4-6) evidence suggest that ventilation with increased pressure/volume stimulates pulmonary and systemic release of pro-inflammatory mediators. It is, however, uncertain whether this phenomenon also occurs in healthy subjects *in vivo*, or whether it only occurs in pre-injured lungs (7, 8). Support for the latter concept came, in part, from studies in which it was shown that injurious ventilation strategies had no effect on serum or bronchoalveolar lavage (BAL) tumor necrosis factor (TNF) levels in rats (7, 9) and in patients (10). In previous *in vivo* studies, elevated serum TNF levels in response to ventilation were only observed if the lungs had previously been instilled with either endotoxin (11, 12) or hydrochloric acid (8). Due to the assumed central role of TNF in the immune response organization (13), in our previous studies we focused on that cytokine only. However, recent evidence from perfused mouse lungs showed that, in response to mechanical ventilation, interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2) are produced in greater abundance than TNF (14).

MIP-2 is a rodent homologue to the major human C-X-C chemokine IL-8, a potent chemotactic cytokine for neutrophilic granulocytes. High intra-alveolar levels of MIP-2 result in increased neutrophil and macrophage accumulation in the lungs, a characteristic of acute lung injury (15).

In ARDS patients, ventilation with higher tidal volumes was associated with higher plasma IL-6 levels (4, 5), and these plasma IL-6 levels correlated with multiorgan failure (16). In addition, IL-6 has been described as an efficient predictor of outcome of ARDS (17). All of these findings suggest that IL-6 and MIP-2 are two important pro-inflammatory mediators that might contribute to injury in lungs subjected to injurious ventilation strategies. In the present study, we therefore re-examined serum and BAL samples acquired from our previous study (7) to test the hypothesis that IL-6 and MIP-2 are released in response to injurious ventilation strategies in vivo.

METHODS

The study was approved by the local Animal Committee of the Erasmus Medical Center Rotterdam. Care and handling of the animals were in accordance with the European

Community guidelines (86/609/EC). The studies were performed in male Sprague-Dawley rats of bodyweight 220-330 g (Harlan CPB, Zeist, The Netherlands).

The different experimental groups and preparation techniques have been described previously (7). Briefly, animals were anesthetized with 65% nitrous oxide/35% oxygen and either 2% isoflurane or 2% enflurane. Subsequently, a sterile polyethylene catheter (0.8 mm outer diameter) was inserted into a carotid artery for drawing arterial blood samples. Thereafter, a sterile metal cannula was inserted into the trachea. After these surgical procedures gaseous anesthesia was discontinued, and anesthesia was continued with 60 mg/kg pentobarbital sodium, i.p. hourly (Nembutal; Algin, Maassluis, The Netherlands).

In the non-ventilated control animals, a broncho-alveolar lavage (BAL) was performed with saline (32 ml/kg heated to 37 °C) and 4 ml of heparinized blood was taken from the arterial line. The animals were then killed by an overdose of pentobarbital sodium.

In all ventilated animals muscle relaxation was induced by pancuronium bromide 2 mg/kg, i.m. hourly (Pavulon; Organon Teknika, Boxtel, The Netherlands) followed by immediate connection to a ventilator. Body temperature was kept within normal range by means of a heating pad. The animals were mechanically ventilated with a Servo Ventilator 300 (Siemens Elema, Solna, Sweden) in a pressure-constant time-cycled mode and a fractional inspired oxygen tension of 1.0.

Animals were ventilated with different pressures for varying times as shown in Table 1. At the end of the study period, BAL was performed and heparinized blood was taken from the arterial line in the same way as described for the non-ventilated control animals. The animals were then killed by an overdose of pentobarbital sodium.

Blood and BAL samples were centrifuged at 400 *g* for 10 min to remove cells and cellular debris, snap frozen in liquid nitrogen and stored at -80 °C until further analysis. From each animal several samples were obtained; thereafter each individual measurement was done using a sample that had not been previously thawed, in accordance with good laboratory practice (18).

IL-6 and MIP-2 measurements

Rat IL-6 and MIP-2 were assessed in blood and BAL supernatant using the rat specific enzyme-linked immunosorbent assay (ELISA) obtained from Endogen (Endogen Inc., Woburn, MA, USA) or Biosource (Biosource Europe S.A., Nivelles, Belgium), respectively.

Statistical analysis

Data in the table are expressed as mean ± standard deviation (SD) in the figures as mean ± standard error of mean (SEM). Since several measurements were below the detection level of the ELISAs, which results in a standard deviation of 0, we chose to analyze the data using non-parametric statistical methods. All data were analyzed with SPSS 10.0 exact tests 7.0 for Windows (SPSS Inc. Chicago, IL, USA). The omnibus hypothesis was tested by the Kruskal-Wallis test and after rejection at $p<0.05$ was followed by one-sided Mann-Whitney tests, testing for the hypothesis that cytokine levels were elevated compared to the control groups. The α-error in the multiple hypothesis testing was adjusted by the Bonferroni-Holm procedure. Statistical significance was assumed at $p<0.05$.

RESULTS

Table 1. Overview of the different experimental groups.

	PIP/PEEP *(cm H_2O)*	*Time on ventilator (min)*	*PaO_2* *(kPa)*	*BAL protein content (mg/ml)*	*n*
	13/3	90	76.5±7.5	0.30±0.19	13
	13/3	240	72.3±8.1	0.29±0.11	13
	32/6	90	68.9±6.7	0.31±0.19	10
	32/6	240	68.8±15.1	0.66±0.47	10
	32/0	90	9.9±3.2	1.91±0.62	10
	32/0	240	8.9±1.9	2.12±0.45	10
Control	Spontaneous breathing	—	—	0.21±0.10	19

n, number of rats

During ventilation with 13 cm H_2O/3 cm H_2O (Peak inspiratory pressure (PIP)/PEEP) or with 32 cm H_2O/6 cm H_2O for 90 min or for 240 min the PaO_2 levels remained above 67 kPa, while ventilation with 32 cm H_2O without PEEP resulted in severe decrease of oxygenation and elevated protein BAL content (Table 1). In lungs ventilated with high PIP and no PEEP (32/0), serum IL-6 levels were increased in the serum after 90 and 240 min, but not in the BAL (Fig. 1a,b). There was no increase in BAL IL-6 levels in any of the groups (Fig. 1b). Analysis of the MIP-2 levels produced different results. Here, ventilation with high PIP levels led to increased serum MIP-2 levels after 90 min with a PEEP of 6 cm H_2O (32/6), and after

240 min in the absence of PEEP (32/0) (Fig. 2a). The MIP-2 BAL levels were increased in all ventilated groups (Fig. 2b), suggesting that mechanical ventilation as such was sufficient to induce this response.

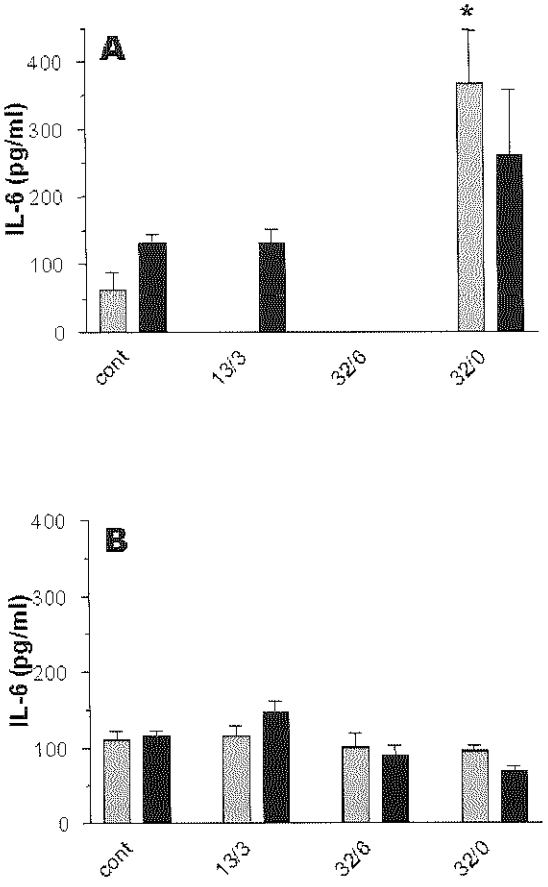

Figure 1.
Concentration of IL-6 serum (A) or BAL (B) after 90 min (grey bars) and 240 min (black bars) ventilation with the following PIP/PEEP levels in cm H_2O: 13/3 (n=13), 32/6 (n=10) or 32/0 (n=10). Control (cont) animals (n=19) were untreated. All data are mean ± SEM. * $p<0.01$ vs control.

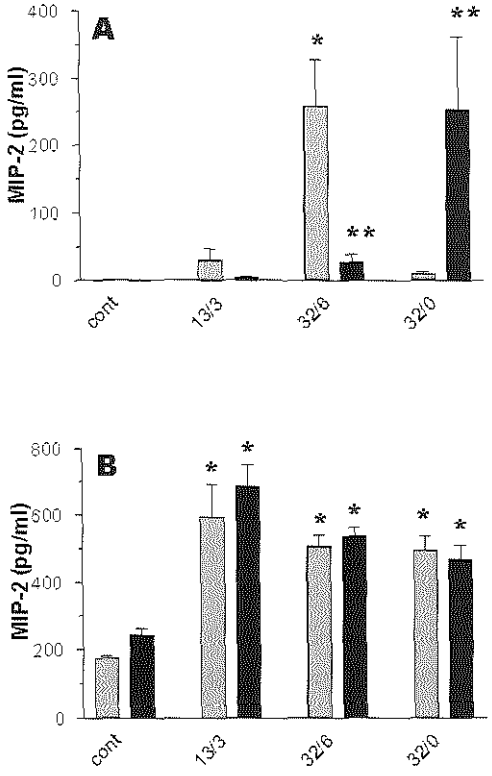

Figure 2.
Concentration of MIP-2 serum (A) or BAL (B) after 90 min (grey bars) and 240 min (black bars) ventilation with the following PIP/PEEP levels in cm H_2O: 13/3 (n=13), 32/6 (n=10) or 32/0 (n=10). Control (cont) animals (n=19) were untreated. All data are mean ± SEM.
* $p<0.01$ vs control, **, $p<0.05$ vs control.

DISCUSSION

There is considerable experimental and clinical evidence that ventilation with increased volumes or pressures may promote the release of pro-inflammatory cytokines. However, whether such a response can be induced in healthy subjects remains unclear. IL-6 and MIP-2

are two potent pro-inflammatory mediators that in response to high pressure ventilation of isolated perfused lungs are produced in greater quantities than is, for instance, TNF (7, 12, 14). We therefore hypothesized that IL-6 and MIP-2 would be better indicators of ventilation-induced cytokine release than TNF. The present data confirm this hypothesis and show for the first time that ventilation can trigger cytokine release in healthy animals.

Systemic and alveolar cytokine levels were analyzed after 90 min or 240 min of ventilation with 32 cm H_2O PIP. In the absence of PEEP, systemic IL-6 levels were increased under any of these conditions. Application of PEEP prevented the IL-6 release at ventilation with 32 cm H_2O PIP.

These observations are comparable to those in isolated perfused mouse lungs, where ventilation-induced cytokine release was also observed under conditions that did not cause major lung damage (1, 3). In line with this, MIP-2 serum levels were elevated in the serum of rats that had been ventilated for 90 min with 32 cm H_2O PIP/6 cm H_2O PEEP, a time point at which according to the BAL protein concentrations and the arterial oxygenation no severe lung injury was present.

To explain the ventilation-induced release of mediators such as IL-6 and MIP-2 in non-injured lungs, it has been suggested that ventilation with high volumes results in overstretching of alveolar units which in turn starts up pro-inflammatory signaling cascades among them activation of NF-□B (14, 19). Subsequently, a number of genes containing NF-κ B response elements (e.g. IL-6 and MIP-2) become activated, whereas those that lack this sequence remain silent (14). The current knowledge on the mechanisms of ventilation-induced mechanotransduction has recently been summarized (20-23). All of these findings suggest that ventilation-induced mediator release can occur independent from physical lung injury.

However, there is also evidence that, in addition to overstretching, the structural lung damage caused by injurious ventilation strategies is another factor that can aggravate or initiate mediator release from the lungs. In the present study, this is illustrated by the protective effect of PEEP on IL-6 release in lungs ventilated with 32 cm H_2O, where PEEP not only prevented the development of tissue damage but also the systemic release of IL-6. This finding is corroborated by previous reports that showed excessive release of mediators in the absence of PEEP in isolated non-perfused rat lungs (2), in isolated perfused mouse lungs (24) and in pre-injured rats *in vivo* (8). The protective effect of PEEP in these models is most

likely explained by the reduction in shear forces that occur during repeated opening and closure of alveolar units in the absence of PEEP, a process that has been termed atelectotrauma (25). On the other hand, it should be noted that zero PEEP alone is not sufficient to elicit mediator release if end-inspiratory pressures are low, as demonstrated in perfused mouse lungs (PIP/PEEP 10/0 cm H_2O) (24) and in humans (16/0 cm H_2O) (10).

In contrast to IL-6, alveolar MIP-2 levels appeared to be very sensitive to ventilation itself, i.e. simply ventilating the animals with normal pressures (e.g. 13/3) caused a significant elevation in BAL MIP-2 levels which, however, were not further augmented by high distending pressures (Fig. 2b). Because in the same BAL samples TNF levels were very low and IL-6 concentrations were similarly low (Fig. 1), we conclude that the increase in MIP-2 was most probably not due to LPS contamination. The same observations, i.e. that mechanical ventilation alone is sufficient to raise alveolar MIP-2 levels in rats (in that case ventilated with 10 ml/kg), have also been reported by Kotani and colleagues (26). Thus, it appears that alveolar MIP-2 is rapidly activated in case of mild stress such as mechanical ventilation or heat (27). The role of MIP-2 during mild stress remains to be elucidated. Nevertheless, our findings suggest that in future studies on ventilation-induced lung injury it will be important to include both IL-6 and MIP-2. This conclusion is corroborated by clinical studies with ARDS patients, in which ventilation with low tidal volumes led to significantly reduced plasma levels of IL-6 (4-6) and IL-8 (the human analogue to MIP-2) (6), but not of TNF (4).

In summary, the present findings demonstrate that in healthy rats aggressive ventilation strategies alone are sufficient to stimulate release of IL-6 and MIP-2 within a short time. Of note, such mediator release not only occurred into the alveolar space, but was also observed in the systemic circulation, suggesting that injurious ventilation strategies may initiate or at least boost not only local but also systemic inflammatory complications in ventilated patients. Finally, this mediator activation by mechanical ventilation was already observed after a relatively short period.

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Chapter 5

Ventilation-induced activation of the mitogen activated protein kinase pathway

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ABSTRACT

Mechanical ventilation of patients can be a life-saving treatment, but also imposes additional stress on the lung. Mitogen activated protein kinases (MAPK) represent a family of protein kinases that become phosphorylated and activated by many different forms of stress. Using Western blot analysis, we analyzed the effects of high distending pressure ventilation on the activation of the MAPK ERK-1/2, JNK and p38 kinase, and on the MAPK-activated transcription factors c-Jun, Elk-1 and ATF-2. In adult rats, ventilation with high pressure (45/10 peak inspiratory pressure/positive end-expiratory pressure in cm H_2O) for 30 or 60 min did not affect arterial oxygenation, but resulted in enhanced phosphorylation of ERK-1/2, JNK, c-Jun, Elk-1 and ATF-2 compared to normally ventilated (13/3) rats. The activation of ERK-1/2 and JNK was located to cells resembling alveolar type II cells. In addition, high pressure ventilation enhanced phosphorylation of IκB–α and nuclear translocation of the transcription factor NF–κB. In isolated perfused mouse lungs, the MEK inhibitor U0126 prevented the ventilation-induced activation of ERK-1 and Elk-1, but had no effect on the ventilation-induced cytokine release. We conclude that mechanical ventilation triggers specific signaling pathways such as the MAP-kinase and the NF-κB pathway which may contribute to pulmonary inflammation and proliferation.

INTRODUCTION

Mechanical ventilation is a life-saving treatment, but if performed with elevated transpulmonary pressures imposes additional stress on the lung. Depending on the extent of the physical forces applied, this stress may lead to activation of pulmonary cells through mechanotransduction presumably initiated by alveolar overdistension [1, 2] or to rupture of membranes and tissue destruction [3]. In support of the notion that ventilation can activate specific signaling pathways, recently we demonstrated activation of the transcription factor NF-κB and release of pro-inflammatory mediators in the absence of gross tissue damage [4-6].

Besides NF-κB, likely candidates for stress-activated signaling cascades are the mitogen-activated protein kinase- (MAPK) dependent pathways, which can be activated by irradiation, osmotic stress, growth factors or strain [1, 7]. MAPK are a family of proline-targeted serine-threonine kinases that transduce environmental stimuli to the nucleus. Mammals express at least four distinctly regulated groups of MAPK: extracellular signal-related kinases (ERK)-1/2, c-Jun amino-terminal kinases (JNK1/2/3) also know as stress-activated protein kinases, p38 kinase and ERK-5 [8]. One major function of MAPK is activation of transcription factors such as ETS like protein-1 (Elk-1), c-Jun, c-Fos, and activating transcription factor-2 (ATF-2) which control a wide variety of genes, many of which are involved in the regulation of inflammation and proliferation. For instance, members of the c-Jun family together with c-Fos and ATF-2 form homo- or heterodimers called AP-1 that activate many different pro-inflammatory genes [9]. Elk-1 cooperatively interacts with the serum response factor (SRF) and binds to the serum response elements (SRE) in the promoter region of various genes, including c-fos and early growth response-1 (egr-1), and triggers their gene expression [10, 11]. However, after phosphorylation by ERK-2, Elk-1 may also activate genes independently from SRF and SRE, as shown for TNF and the chemokine 9E3/cCAF [12, 13]. ATF-2 can combine with c-Jun, and activate the genes for c-jun, TNF and E-selectin [9, 12, 14].

Thus, MAPK are activated by various forms of extracellular stress and might serve an important role in the cellular responses to ventilation with elevated pressures. However, the effect of ventilation with high distending pressures on MAPK is unknown. To this end, rats were ventilated with normal and high peak-inspiratory pressures (PIP) and lung damage was minimized by application of positive end-expiratory pressure (PEEP) [15, 16]. Activation of

MAPK was analyzed by Western Blot and immune immunohistochemistry. To examine some of the consequences of MAPK activation, we analyzed the activation of transcription factors that are known to be controlled by MAPK. Furthermore, to show that these transcription factors are controlled by MAPK, we used perfused mouse lungs to study the effect of the MEK-inhibitor U0126 on the phosphorylation of ERK-1 and Elk-1 and on the cytokine release. Finally, to confirm previous findings in perfused mouse lungs *in vivo* [4], we investigated the activation of NF−κB in rats *in vivo*.

MATERIALS AND METHODS

The study was approved by the local Animal Committee of the Erasmus University Rotterdam. Male Sprague-Dawley rats (body weight 220-330 g) were from Harlan (CPB, Zeist, The Netherlands), Female BALB/C mice (20-23 g) from the breeding house of the Research Center Borstel. Care and handling of the animals were in accord with the NIH guidelines.

Ventilation of rats in vivo

The preparation techniques have been described previously [16, 17]. Briefly, animals were anesthetized with 65% NO_2 /35% O_2 and 2% isoflurane. Subsequently, a sterile polyethylene catheter was inserted into a carotid artery for drawing arterial blood samples, and monitoring blood pressure. Thereafter, a sterile metal cannula was inserted into the trachea. After these surgical procedures, gaseous anesthesia was discontinued and anesthesia was continued with 60 mg/kg pentobarbital sodium i.p. (Nembutal; Algin, Maassluis, The Netherlands). Subsequently, muscle relaxation was induced by pancuronium bromide 2 mg/kg, i.m. (Pavulon; Organon Teknika, Boxtel, The Netherlands), and ventilation was initiated with a Servo Ventilator 300 (Siemens Elema, Solna, Sweden) in a pressure-constant time-cycled mode and an FiO_2 of 1.0.

Rats (n=4 per group), were mechanically ventilated at a frequency of 30 breaths/min with normal pressure (13/3; PIP/PEEP in cm H_2O) or with high pressure (45/10) for 30 or 60 min. At the end of the study period, heparinized blood was taken from the arterial line, and animals were then killed with an overdose of pentobarbital. The unventilated control group was killed immediately after tracheotomy in an identical way. The thorax was opened and lungs were collected sterile, snap frozen and stored. For immunohistochemistry, the thorax

was opened, lungs were removed en bloc and filled with HOPE solution [18, 19], subsequently the lung was inflated with a PEEP level of 10 cm H_2O, the trachea was clamped and the lungs were stored in Hope solution at 4° C until further analysis.

Immunohistochemistry

HOPE-fixed, paraffin-embedded specimen were prepared like previously described [18, 19]. 4µm thick sections were cut, mounted on Superfrost+ slides (Menzel-Gläser, 041300, Germany) and deparaffinized as described elsewhere [18, 19]. Negative controls were included in every staining series as well as positive reference sections to ensure even results. Samples were pretreated in 25% H_2O_2 for 30 min at ambient temperature

The primary antibodies were then applied each in a dilution of 1/100 in phosphate buffered saline (PBS) for 1hr at ambient temperature. Slides were washed in PBS, twice, 1min for each washing step. Secondary antibody (donkey anti rabbit, conjugated with alkaline phosphatase and absorbed against mouse, dianova 44311, Germany) was applied in a dilution of 1/100 in PBS for 30 min. at ambient temperature. Slides were washed twice (1min each) in PBS.

Color reaction was then performed by incubation with new fuchsine solution according to the manufacturer's protocol (Dako, K 0624, Germany). Color reaction was complete within 10 min. Counterstaining was achieved by Mayer's hemalum; slides were mounted by using Kayser's glycerin-gelatine and photographed.

Isolated perfused mouse lung

The mouse lungs were prepared and perfused essentially as recently described [4, 6, 20]. Briefly, lungs were perfused in a non-recirculating fashion through the pulmonary artery at a constant flow of 1 ml/min resulting in a pulmonary artery pressure of 2 to 3 cm H_2O. As a perfusion medium we used RPMI medium lacking phenol red (37°C). Under control conditions, the lungs were ventilated with room air by negative pressure (–3 cm H_2O to –10 cm H_2O) at a rate of 90 breaths/min resulting in a tidal volume (V_T) of about 200 µl. Artificial thorax chamber pressure was measured with a differential pressure transducer (DP 45-24; Validyne, Northridge, CA), and the airflow rate was measured with a Fleisch-type pneumotachograph tube connected to a differential pressure transducer (DP 45-15, Validyne). Arterial pressure was continuously monitored by means of a pressure transducer (Isotec Healthdyne, Irvine, CA) that was connected to the cannula ending in the pulmonary artery. All data were transmitted to a computer and analyzed with Pulmodyn software (Hugo Sachs

Elektronik, March Hugstetten, Germany). V_T was derived by integration of the flow rate, and the data were analyzed by applying the formula: $P = 1/C \cdot V_T + R_L \cdot dV/dT$, where P is chamber pressure, C is pulmonary compliance and R_L lung resistance.

Western blot analysis

Frozen lungs were powdered with a pestle in the constant presence of liquid nitrogen. Aliquots of the lung powder were lysed and homogenized in a buffer (50 mM Tris-Cl, pH 6.8, 150 mM NaCl, and 1% Triton X-100) containing Pefablock (1mM), aprotinin (1µg/ml), pepstatin (1 µg/ml), leupeptin (1 µg/ml), NaF (1mM), Na_3VO_4 (1 mM), ß-Glycerolphosphate (1 mM). After 20 min on ice, the lysates were collected by pelleting the cellular debris for 15 min at 13,000 x g. Total protein content was determined by a commercially available test (Pierce, Rockford, IL, USA).

An equal amount of protein (60µg/slot) was size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Dassel, Germany) and then immunoblotted with primary antibodies (New England Biolabs (NEB), Frankfurt, Germany) and horseradish peroxidase-conjugated secondary antibodies (NEB). Detection of the bound antibody with LumiGLO chemiluminescent substrate was performed according to the manufacturer (NEB). The densitometric analysis was performed with OPTIMAS 6.2 software (Optimas Corporation, Bothel, WA, USA).

Immunoprecipitation

Aliquots of the mouse lung powder were lysed in 500 µl of cold lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM Na_3VO_4, 0.2 mM PefaBloc, 1% NP-40) by constant agitation for 30 min at 4°C by end over end rotation. To disperse larger aggregates the raw lysate was sonicated 3 x 5 sec (Branson, Switzerland). The final lysate was collected by centrifugation (16 000 x g, 4°C, 15 min). Total protein content was determined by a commercially available test (Pierce, Rockford, IL, USA). For the precipitation, 5 µg of first antibody and 500 µg total protein in 2x immunoprecipitation buffer (20 mM Tris pH 7.4, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA pH 8.0, 0.4 mM Na_3VO_4, 0.4 mM PefaBloc, 1% NP-40) were incubated overnight at 4°C by end over end rotation. The immunocomplex was collected by incubation with Protein G Plus Agarose (Santa Cruz Biotechnology, Inc) with constant agitation for 1 hr at 4°C and centrifugation for 4 min (16 000 x g, 4°C). The pellet was washed three times with 1 x immunoprecipitation buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA

pH 8.0, 0.2 mM Na_3VO_4, 0.2 mM PefaBloc, 0.5% NP-40). The precipitate was resuspended in electrophoresis sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 5% glycerol, 0.006% bromophenol blue, 2% ß- mercaptoethanol), boiled for 5 min, centrifuged and the supernatant loaded onto a SDS-PAGE gel, electrophoresed and blotted as described for western blotting.

Electrophoretic mobility shift

The preparation of nuclear extracts and electrophoretic mobility shift assays were performed as described previously [4].

Table 1: Oxygenation, mean arterial pressure, serum enzymes and serum creatinine

	13/3			45/10		
	5 min (n=8)	30 min (n=8)	60 min (n=4)	5 min (n=8)	30 min (n=8)	60 min (n=4)
pO_2 (mmHg)	587±23	602±49	605±78	592±34	607±28	588±38
pCO_2 (mmHg)	30±4	37±7	34±9	30±4	34±3	33±4
MAP (mmHg)*	151±17	159±11	152±18	99±17	126±14	137±12
		(n=4)	(n=4)		(n=4)	(n=4)
ASAT (U/L)	—	61±14	48±13	—	52±12	73±21
ALAT (U/L)	—	23±3	20±3	—	23±2	24±2
creatinine (mg/L)	—	0.7±0.2	0.7±0.1	—	0.7±0.2	0.8±0.2

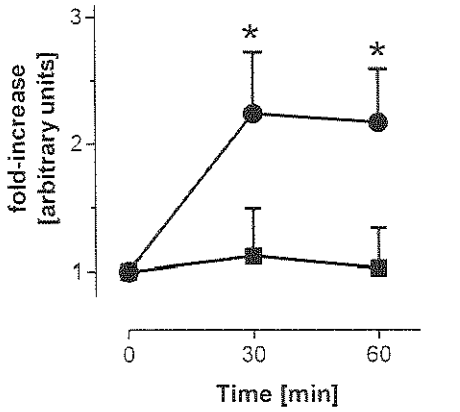
*, $p<0.05$: 13/3 vs 45/10 by repeated measurement analysis (SPSS). The data in unventilated control animals for serum aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and creatinine were 82±20 U/L, 22±4 U/L and 0.9±0.2 mg/L, respectively. MAP, mean arterial pressure. n, number of animals.

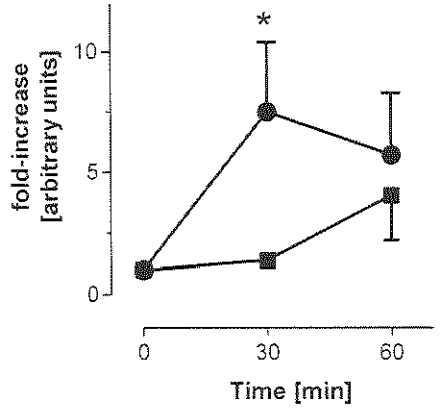
Statistical analysis

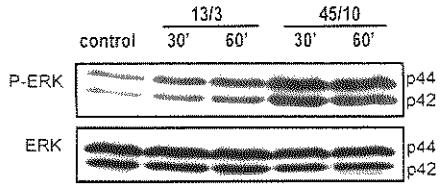
The intensity of the bands in the blots was quantified and expressed as a ratio of a value from an unventilated control animal. All data were log transformed and analyzed for the difference between 13/3 and 45/10 by paired (to analyze the data from one gel together) one-sided t-tests. Statistical significance was assumed at $p<0.05$.

RESULTS

Ventilation with 13 cm H_2O /3 cm H_2O (PIP/PEEP) or 45 cm H_2O /10 cm H_2O led to tidal volumes of 18 ml/kg and 48 ml/kg, respectively [21]. The 60 min of ventilation with 45/10 cm H_2O were well tolerated as indicated by the normal blood oxygenation (Table 1). As expected, mean arterial pressure decreased under these conditions. The serum transaminase and creatinine levels were not different in ventilated and nonventilated animals (Table 1).







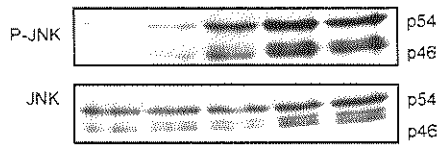


Figure 1
Time course of ERK-1/2 phosphorylation in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. ERK was analyzed by immunoblot, using antibodies specific for the phosphorylated or the unphosphorylated form of ERK. Shown is a representative immunoblot and densitomteric data (mean of p44 and p42). The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. *, $p< 0.05$ vs 13/3. p42 and p44 represent ERK-1 and ERK-2, respectively.

Figure 2
Time course of JNK phosphorylation in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. JNK was analyzed by immunoblot, using antibodies specific for the phosphorylated or the unphosphorylated form of JNK. Shown is a representative immunoblot and densitomteric data (p54). The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. After 30 min of ventilation with normal pressure (square) the fold increase over baseline was 1.40±0.31. *, significantly ($p < 0.05$) larger than 13/3 at that time point. P54 and p46 represent JNK isoforms.

MAPK

Under no condition, differences were detected in the expression of the native unphosphorylated forms of ERK-1/2 and JNK (Fig. 1, Fig. 2). Ventilation of rats for 30 or 60 min with 13/3 cm H_2O had no effect on ERK-1/2 phosphorylation, whereas ventilation with 45/10 cm H_2O increased ERK-1/2 phosphorylation about 2-fold (Fig. 1). There was no difference in phosphorylation between ERK-1 and ERK-2. Of the two JNK isoforms analyzed, p54 (Fig. 2) showed more consistent results than p46, which however showed the same pattern as p54. Phosphorylation of JNK was not enhanced after ventilation with 13/3 cm H_2O for 30 min, but was about 4-fold enhanced after ventilation for 60 min, suggesting that ventilation by itself may be a stimulus for JNK-activation. Ventilation with 45/10 cm H_2O resulted in a 7-fold activation of JNK after 30 min. Native p38 kinase was present in similar amounts under all conditions. Both modes of ventilation appeared to increase phosphorylation of p38, but ventilation with 45/10 cm H_2O was not different from ventilation with 13/3 cm H_2O (Fig. 3).

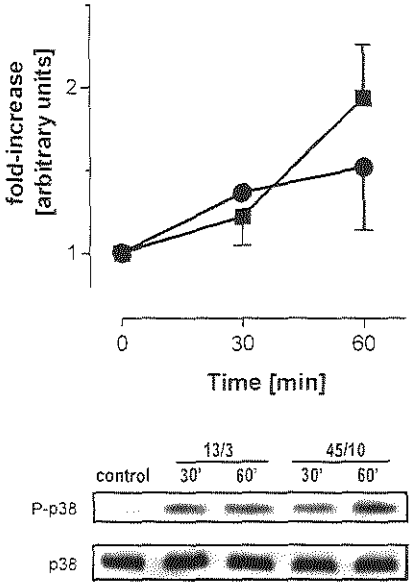


Figure 3
Time course of p38 phosphorylation in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. p38 phosphorylation was analyzed by immunoblot, using phosphospecific c-Jun antibodies. Shown is a representative immunoblot and densitometric data. The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. After 30 min of ventilation with 45/10 cm H_2O (circle) the fold increase over baseline was 1.36±0.05.

Immunohistochemistry was performed to identify the cell type in which overinflation led to phosphorylation of MAPK. Because in preliminary experiments, the antibodies used for the Western-Blots did not appear to work with classical formalin fixation, we employed the novel HOPE fixation technique, a method which was developed as a less denaturing alternative to

conventional fixation [18]. Using this method, we were able to obtain positive staining with antibodies specific for the phosphorylated forms of ERK, JNK and p38 (Fig. 4).

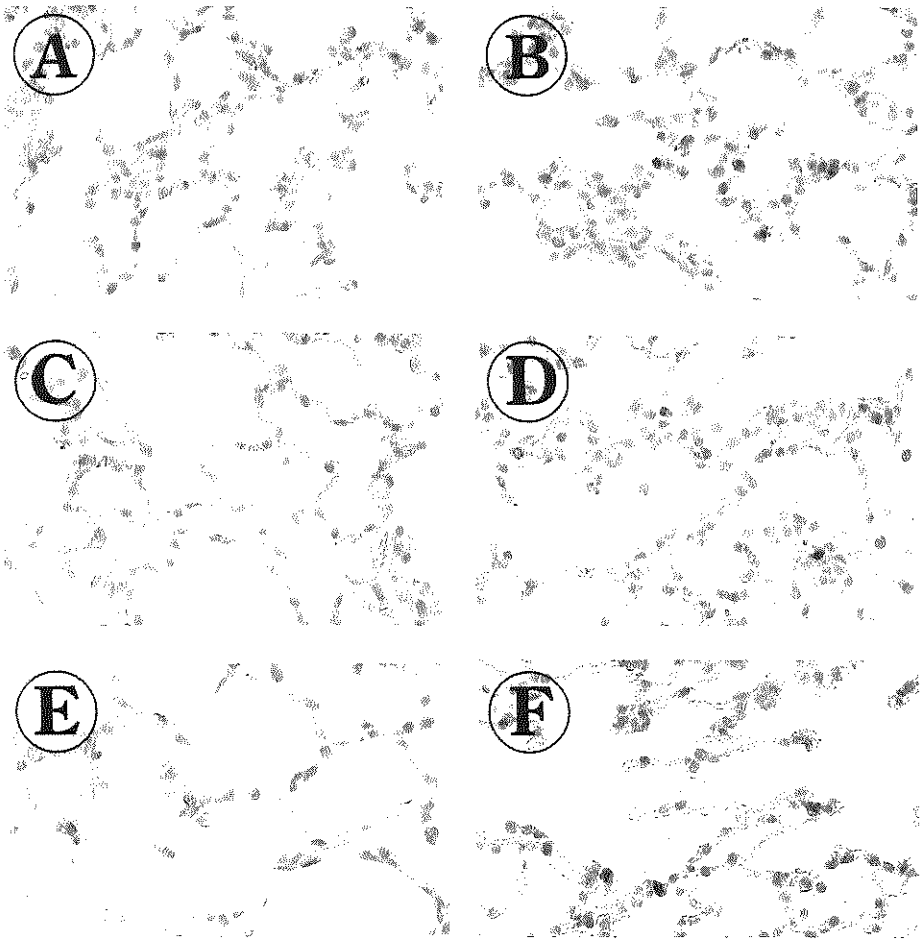


Figure 4
Activation of MAPK in rat lungs *in vivo*. Rats were either mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O; Panels **A**,**C**,**E**) or with high pressure (45/10; Panels **B**,**D**,**F**) for 60 min. The immunohistochemical staining (red) with antibodies specific for the phosphorylated forms of ERK (top panels A and B), JNK (middle panels C and D) and p38 (bottom panels E and F) shows that overinflation activated ERK and JNK predominately in alveolar epithelial type II cells. X 800.

P-ERK and P-JNK were only weakly detectable in lungs ventilated for 60 min with 13/3 cm H_2O (Fig. 4A and C), but the signal was clearly increased in cells whose size, shape and location resembled alveolar epithelial type II cells, but sometimes also in endothelial cells, of lungs ventilated with 45/10 cm H_2O (Fig. 4B and D). Similar observations were made after

30 min of ventilation (data not shown). However, at both time points the distribution of P-ERK or P-JNK positive cells was heterogeneous and activation was not observed in all type II cells. P-p38 was also mostly detected in cells resembling alveolar epithelial type II cells, but there was no difference in lungs ventilated with either 13/3 cm H_2O or 45/10 cm H_2O (Fig. 4E and F). Again, P-p38 was only found in a fraction of the type II cells. For all MAPK, the possibility of activation in alveolar epithelial type I cells cannot be excluded.

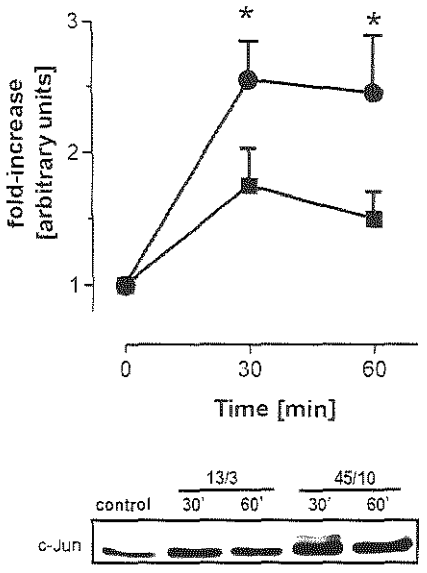


Figure 5
Time course of c-Jun expression in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. c-Jun was analyzed by immunoblot, using specific c-Jun antibodies. Shown is a representative immunoblot and densitomteric data. The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. *, significantly ($p < 0.05$) larger than 13/3 at that time point.

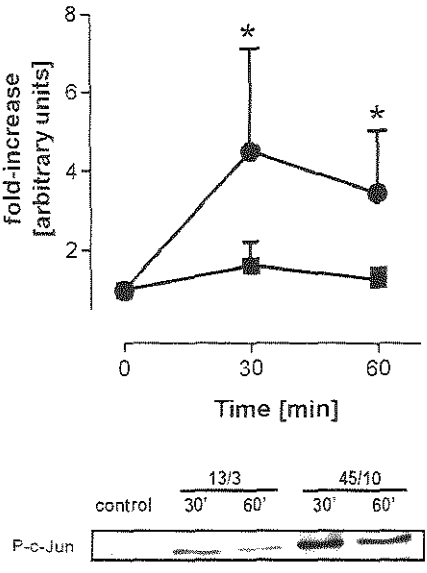


Figure 6
Time course of c-Jun phosphorylation in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. c-Jun phosphorylation was analyzed by immunoblot, using phosphospecific c-Jun antibodies. Shown is a representative immunoblot and densitomteric data. The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. *, significantly ($p < 0.05$) larger than 13/3 at that time point.

MAPK-activated transcriptions

To investigate some of the intracellular consequences of the activation of ERK and JNK, we investigated the activation of the transcription factors c-Jun, Elk-1 and ATF-2. c-Jun belongs to the immediate early genes that are rapidly formed in response to a variety of stimuli. In line with this, ventilation with 45/10 cm H_2O increased both the expression of native c-Jun (Fig. 5) as well as its phosphorylation (Fig. 6). In addition, ventilation with 45/10 cm H_2O elicited a 6-fold increase in the phosphorylation of Elk-1 (Fig. 7) and a nearly 3-fold increase in the phosphorylation of ATF-2 (Fig. 8). No differences were detected in the expression of the native forms of Elk-1 or ATF-2.

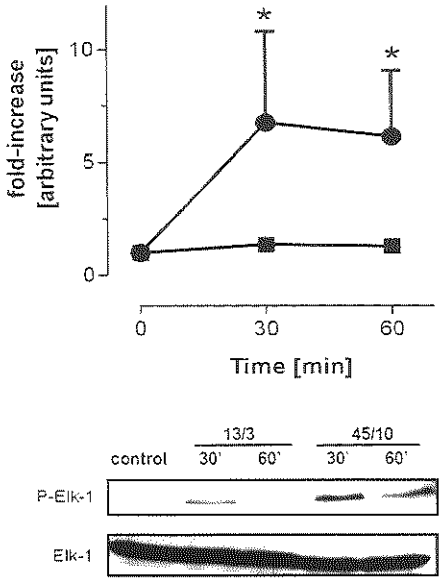


Figure 7
Time course of Elk-1 phosphorylation in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. Elk-1 was analyzed by immunoblot, using antibodies for specific for the phosphorylated or the unphosphorylated form of Elk-1. Shown is a representative immunoblot and densitomteric data. The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. After 30 and 60 min of ventilation with normal pressure (square) the fold increase over baseline was 1.38±0.21 and 1.30±0.11, respectively. *, significantly ($p < 0.05$) larger than 13/3 at that time point.

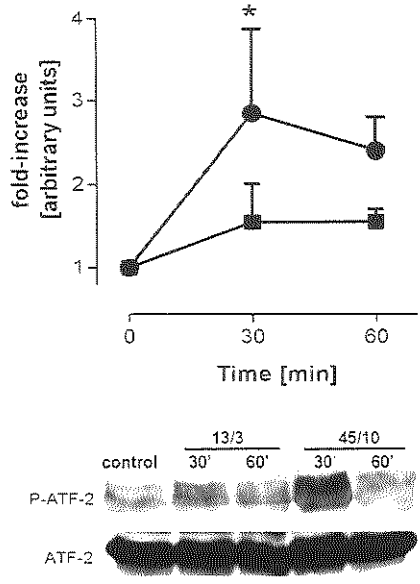


Figure 8
Time course of ATF-2 phosphorylation in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. ATF-2 was was analyzed by immunoblot, using antibodies for specific for the phosphorylated or the unphosphorylated form of ATF-2. Shown is a representative immunoblot and densitomteric data. The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. *, significantly ($p < 0.05$) larger than 13/3 at that time point.

NF–κB

Ventilation with 45/10 cm H_2O rapidly increased phosphorylation of IκB–α (Fig. 9). Phosphorylation is known to lead to degradation of IκB–α, which in turn facilitates nuclear translocation of NF–κB. In line with this, we found increased amounts of NF-κB in the nuclear extracts of rats ventilated with 45/10 cm H_2O (Fig. 10).

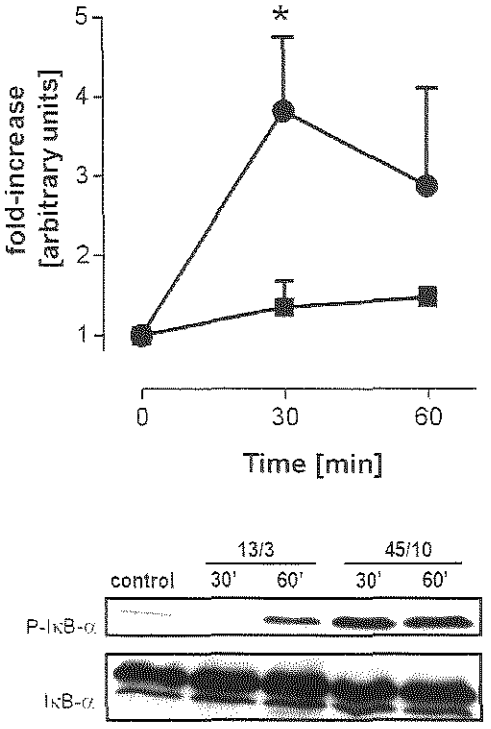


Figure 9
Time course of IκB-α phosphorylation in lung homogenates. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3: PIP/PEEP in cm H_2O, square) or with high pressure (45/10, circle) for 30 or 60 min. IκB-α was analyzed by immunoblot, using antibodies for specific for the phosphorylated and the unphosphorylated form of IκB-α. Shown is a representative immunoblot and densitomteric data. The values are shown as fold increase over the unventilated control and represent the mean ± SEM from four animals. *, significantly ($p < 0.05$) larger than 13/3 at that time point.

ERK-1, Elk-1 and Cytokine release in isolated perfused mouse lungs

Finally, we investigated some of the consequences of the ventilation-induced activation of MAPK. To his end, we used isolated perfused mouse lungs, a model which allows precise control over ventilation and perfusion, and in which confounding factors such blood cells or enervation can be excluded [6]. Lungs were ventilated with either –10/–3 or –25/–3 cm H_2O, resulting in tidal volumes of 9 and 32 ml/kg, respectively, as described before [4, 6]. Like *in vivo*, also in perfused mouse lungs ventilation with increased pressures for 60 min resulted in enhanced phosphorylation of ERK-1 (Fig. 11) and JNK (2.8 ± 1.4-fold, n=3, mean±SD). The

activation of ERK, but not that of JNK (data not shown) was completely abolished by pretreatment with the selective MEK-inhibitor U0126 (Fig. 11). At the same time, U0126 also prevented the phosphorylation of Elk-1 (Fig. 11). However, pretreatment with U0126 had no effect on the ventilation-induced release of IL-6 and MIP-2 into the perfusate (Fig. 12).

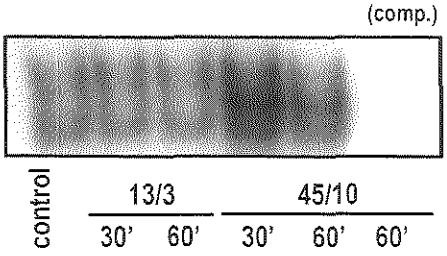


Figure 10
Nuclear factor-κB-translocation by overinflation in lungs from rats. Rats were either left unventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O) or with high pressure (45/10) for 30 or 60 min. NF-κB translocation was determined by electromobility shift assay. The NF-κB band was abolished in the presence of unlabeled ('cold') oligonucleotides (comp, rightmost lane).

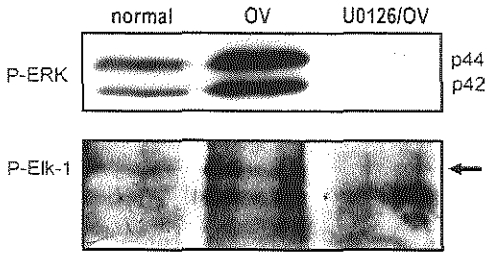


Figure 11
Activation of ERK-1 and Elk-1 in isolated perfused mouse lungs ventilated for 60 min with normal pressure (–10/–3; end-inspiratory pressure/PEEP in cm H_2O) or with high pressure (–25/–3, OV). 10 μM U0126 was added to the perfusate 10 min before switching to overinflation. ERK-1 was analyzed by immunoblot, using antibodies for specific for the phosphorylated form of ERK-1. Elk-1 was analyzed by immunprecipitation using antibodies specific for the phosphorylated form of Elk-1. The P-Elk-1 band is indicated by the arrow; the identity of the other bands is not known. Shown are representative immunoblots from three independent experiments.

DISCUSSION

MAPK are known to become rapidly activated by various forms of stress. The present study shows that this is also true for the mechanical stress caused by ventilation with high distending pressures. A major cell type in which MAPK become activated in response to overinflation appear to be alveolar type II cells, although type I cells cannot be excluded. The fact that overinflation activated MAPK not only *in vivo*, but also in isolated mouse lungs, suggests that this phenomenon is not secondary to leukocyte influx or nervous regulation. The phosphorylation and/or expression of the transcription factors ATF-2, Elk-1 and c-Jun provides evidence that the MAPK were effectively activating their commonly known targets,

as was specifically demonstrated herein for Elk-1. However, our findings also indicate that the ERK-pathway does not contribute to the ventilation-induced release of pro-inflammatory cytokines and chemokines (IL-6, MIP-2), leaving the final physiological consequence of the MAPK activation unknown. In view of the well known contribution of MAPK to cell proliferation [22], we speculate that their preferential activation in type II cells might have bearing on the type II cell hyperplasia that is observed in chronically ventilated individuals [23]. This also raises the question whether the ventilation-induced activation of MAPK may be related to the fibro-proliferative phase of ARDS, in particular to the type II cell hyperplasia commonly observed in these patients [24]. In this regard, in future studies it will also be important to investigate MAPK expression at later time points.

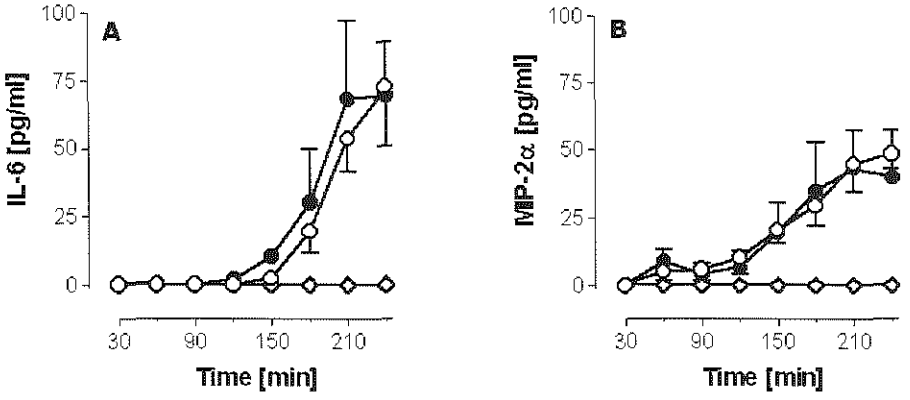


Figure 12
Ventilation-induced alterations in IL-6 and MIP-2α levels in the perfusate of isolated perfused mouse lungs. Lungs were ventilated for 60 min with normal pressure (–10/–3; end-inspiratory pressure/PEEP in cm H_2O) before they were ventilated for another 180 min with normal pressure (open diamonds, n=3), with –25/–3 cm H_2O (open circles, n=5), ventilated with –25/–3 cm H_2O and pretreatment with 10 μM U0126 (closed circles, n=3). Data are mean ± SEM.

Rats were ventilated with either 13/3 cm H_2O or 45/10 cm H_2O. These ventilation modes were chosen to minimize end-expiratory alveolar collapse and subsequent surfactant dysfunction [21]. Previous studies have shown that when animals are ventilated with a PIP of 45 cm H_2O, lung injury can be reduced by applying a PEEP of 10 cm H_2O [15, 16], although at a PIP as high as 45 cm H_2O the PEEP does not completely prevent lung injury, particularly at later time points. Nevertheless, oxygenation as a measure for lung function and serum transaminase activities and creatinine levels as indicators of extrapulmonary

organ injury were not different during ventilation with 45/10 cm H_2O compared to ventilation with 13/3 cm H_2O after one hour (see Table 1). From this, we conclude that during the first 60 min ventilation with 45/10 cm H_2O did not cause severe lung injury (which is also supported by the histological data), so that the activation of MAPK under these conditions is most likely explained by specific mechanotransduction mechanisms rather than by unspecific processes that may originate from damaged cells [3]

At present it is not known how the strain and the stress caused by ventilation is converted into biological signals, but it is reasonable to hypothesize that the initial signal sensed by the cells is stretch. This is supported by the fact that mechanical stretch has been shown to activate ERK and JNK in cardiac myocytes [25], mesangial cells [26], melanocytes [27], pulmonary endothelial cells [28] and L929 cells [29]. Activation of ERK-1/2 (p42/p44) was reported within 10 min after 20% elongation of H441 pulmonary epithelial cells [30]. In A549 cells it was shown that 15% strain activated JNK (stress-activated protein kinase) within 30 min and p38 kinase at later time points (2 h), whereas ERK-1/2 was not activated [31]. These latter results in A549 cells are in contrast to the ventilation-induced activation of ERK in type II cells *in vivo* (Fig. 4B), and indicate that stretching of A549 cells can only partially model the situation of alveolar epithelial type II cells in the whole intact organ.

To our knowledge, so far only one study has identified particular cell types as responders to overinflation *in vivo* [32]. By using in situ hybridization Foda *et al.* showed activation of the extracellular matrix metalloprotein inducer (EMMPRIN), gelatinase A, and gelatinase B in alveolar macrophages, alveolar epithelial cells and endothelial cells from rats ventilated with 20 ml/kg and no PEEP for 4 hours [32]. Among these cells, in the present study activation of MAPK by overinflation was noted particularly in alveolar epithelial type II cells and occasionally in endothelial cells. However, due to the lack of a selective type II cell counterstain, the present data do not completely exclude MAPK activation in other cell types such as type I cells. Previous studies already suggested that type II cells can respond to stretch, in particular those studies showing release of surfactant [33] and IL-8 from stretched alveolar epithelial cells [34]. Indirect evidence for the type II cells' responsiveness to stretch was already provided almost 20 years ago, when it was shown that even one single deep breath is sufficient to release pulmonary surfactant [35]. Whether MAPK contribute to the ventilation-induced surfactant release or IL-8 production is currently not known. However,

since MIP-2 is considered as a murine analogue to IL-8, our negative findings with U0126 treatment suggest by analogy that ERK may not contribute to IL-8 release, too.

In contrast to JNK and ERK-1/2, p38 kinase was not activated by overinflation, suggesting some specificity in the ventilation-induced signaling cascades. A possible explanation for the absence of p38 activation by overinflation could be the recent observation that activation of p38 kinase in response to stretch is regulated differently from JNK and ERK-1/2 [29]. However, we cannot exclude the possibility that p38 becomes activated at time points later than 60 min as was observed in strained A549 cells [31]. Interestingly, phosphorylation of p38 was noted in all ventilated lungs, indicating that the process of mechanical ventilation *per se* might be sufficient to activate p38. A similar finding has been reported for MIP-2 [36, own unpublished observations]. Whether there is a link between p38 and MIP-2 under these conditions, remains to be established, but it is known that p38 contributes to LPS-induced pulmonary MIP-2 production [37].

Given that one major route of MAPK action is through activation of transcription factors, the phosphorylation of Elk-1, c-Jun and ATF-2 by overinflation was expected. From the experiments with the highly specific MEK-inhibitor U0126 [38], we conclude that in response to overinflation Elk-1 is predominantly activated by ERK. Interestingly, Elk-1 is known to regulate c-Fos transcription, which was found to be upregulated in isolated rat lungs ventilated with injurious ventilation strategies [39]. The consistent presence of Elk-1 binding elements in the promoter regions of many immediate early response genes (e.g. c-fos, MKP-1, egr-1) [13] suggests that this pathway may play an important role in the responses to ventilation-induced stress.

The expression of native unphosphorylated c-Jun was increased by overinflation. This may be explained by the fact that the c-Jun promoter binds c-Jun: ATF-2 heterodimers that upon phosphorylation by JNK lead to enhanced c-Jun transcription and subsequent production of c-Jun [9].The enhanced expression of c-jun could, at least partly, explain the increase in P-c-Jun. C-Jun may form homodimers or, together with c-Fos, heterodimers which belong to the AP-1 family of transcription factors, the transcriptional activity of which is further enhanced by phosphorylation of Ser63 and Ser73 on c-Jun by JNK [9]. Therefore, our findings suggest that AP-1 is formed and activated during overinflation. Because AP-1 may also form from C-Jun homodimers, the failure of U0126 to affect ventilation-induced cytokine release does not rule out involvement of AP-1 in this process. AP-1 is well known for controlling a number of

pro-inflammatory genes such as IL-2, IL-5, GM-CSF, IFN-γ and matrix metalloproteinases [9]. Clearly, the role of AP-1 and other transcription factors such as Egr-1 during ventilation with unpyhsiologically high distending pressures deserves further study.

In addition to the MAP kinase pathway, in this study we have shown activation of NF–κB in rats ventilated with high pressures. Nuclear translocation of NF–κB in response to ventilation or stretch has previously been shown by assays in cell culture [40] and in isolated perfused mouse lungs [4], but not *in vivo*. This study also provides evidence that the activation of NF–κB is accompanied by phosphorylation of its inhibitor IκB–α. This phosphorylation is known to lead to ubiquitinylation and subsequent degradation of IκB–α, which is a prerequisite for translocation of NF–κB into the nucleus. NF–κB controls many pro-inflammatory genes and in mouse lungs we have recently shown that only those mediators whose genes possess an NF–κB consensus sequence in their promotor are released in response to overinflation [4]. Taken together, these findings suggest that ventilation might contribute to the activation of NF–κB, as observed in ARDS patients [41].

In summary, we have shown that ventilation with high distending pressures activates ERK-1/2 and JNK in alveolar epithelial type II cells as well as several transcription factors (Elk-1, c-Jun; ATF-2, NF–κB) in as yet unidentified lung cells. These findings suggest that overinflation elicits specific signaling pathways that lead to well-coordinated cellular responses. We hypothesize that these signaling pathways might contribute to the cellular proliferation and inflammation seen in chronically ventilated patients.

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Chapter 6

Ventilation-induced activation of cytokines, ERK and AKT kinase in the presence of exogenous surfactant *in vivo*

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Submitted

ABSTRACT

Alveolar overdistension caused by mechanical ventilation leads to activation of specific intracellular signaling cascades and to the release of pro-inflammatory mediators. The distensibility of a lung is dependent on the amount of active surfactant, which will be lost from the alveoli when lungs are ventilated for longer periods with high distending pressures, so that the alveoli will distend less and stretch will diminish overtime. To study the role of prolonged stretch in healthy lungs, we increased surfactant levels to supra-physiological levels. Surfactant-treated or untreated rats were ventilated for either 30 min or 120 min with a peak inspiratory pressure/positive end-expiratory pressure of 13/3 or 45/10 cmH_2O. We studied the effect of ventilation and surfactant on the phosphorylation of ERK1/2, p38 kinase and AKT kinase, and on serum levels of TNF, IL-6 and MIP-2α. Ventilation with high pressure, with or without additional surfactant, time dependently increased MIP-2α and IL-6 serum concentrations and the phosphorylation of ERK1/2 and AKT. Surfactant pre-treatment significantly increased TNF serum concentrations in overventilated rats. Our findings indicate that the specific signaling and mediator responses induced by prolonged ventilation with high distending pressure are either unaltered or even accentuated by exogenous surfactant.

INTRODUCTION

Mechanical ventilation always bears the risk of regional alveolar overdistension. The resulting increased stretch is now recognized as an important cellular stimulus leading to activation of various intracellular signaling cascades and release of pro-inflammatory mediators (biotrauma) [1, 2]. Many of the pro-inflammatory responses induced by pulmonary overdistension appear to be signaled by activation of NF-κB, such as the release of IL-6, TNF, MIP-2α, MCP-1, or IL-8 [3, 4]. Another signaling pathway that is activated by stretching both in culture [5] and *in vivo* [6] is the ERK pathway with subsequent activation of the transcription factor ELK-1 [6]. However, the physiological relevance of this pathway is less clear, because prevention of ERK activation attenuated stretch-induced IL-8 release in culture [7], but had no effect on ventilation-induced release of IL-6 and MIP-2α in the isolated intact organ [6]. A third pathway, one that has not yet been investigated in the context of ventilation-induced lung injury, is activation of AKT kinase (protein kinase B). The fact that AKT kinase is activated by shear stress [8], suggests that this pathway may also respond to the mechanical stretch generated by ventilation with high pressure/ volume.

Exogenous surfactant is considered as a treatment for acute lung injury [9], a condition that requires continued mechanical ventilation with higher than normal transpulmonary pressures. In isolated perfused mouse lungs surfactant treatment increased the ventilation-induced release of TNF and IL-6 [10], a finding which can be explained by the enhanced distensibility of these lungs after surfactant treatment. When lungs are subjected to high peak inspiratory pressures the endogenous surfactant is squeezed from the alveoli to the distal airways [11] and active surfactant is converted into inactive surfactant by large surface area changes [12], finally leading to reduced pulmonary compliance and stretch of the lung over time. This phenomenon is prevented or at least attenuated by increasing the amount of surfactant to supra-physiological levels. Therefore, surfactant treatment allows studying the effect of longer periods of stretch on the activation of ventilation-induced signaling and mediator responses. Based on these considerations, the present study had two objectives: to extend the experiments with isolated perfused mouse lungs [10] to the level of whole intact animals, and to prolong the period of stretch as compared to previous *in vivo* investigations [6]. To this end we studied the effect of surfactant treatment in combination with high or normal pressure

ventilation on the systemic release of TNF-α, IL-6 and MIP-2α, and on the activation of ERK, p38 kinase and AKT kinase in healthy rats.

MATERIALS AND METHODS

The study was approved by the local Animal Committee of the Erasmus Medical Center Rotterdam. Thirty six male Sprague-Dawley rats (body weight 220-330 g) were obtained from Harlan (CPB, Zeist, The Netherlands). Care and handling of the animals were in accord with the NIH guidelines.

Surfactant pretreated animals

Animals pretreated with surfactant were anesthetized with 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane; Pharmachemie BV, Haarlem, The Netherlands), and tracheotomized. A sterile metal cannula was inserted into the trachea; subsequently the operation area was infiltrated with 30 mg/kg lidocaine (xylocaine; Astra Pharmaceutical BV, Rijswijk, The Netherlands). Exogenous natural surfactant (HL-10, Leo Pharmaceuticals Products, Ballerup, Denmark and Halas Pharma GmbH, Oldenburg, Germany) 400 mg/kg bodyweight (dissolved in 50 mg/ml of saline) was administered through the tracheal cannula over 5 minutes in the spontaneous breathing animals; all animals recovered from anesthesia and breathed spontaneously for the ensuing 60 minutes to allow the instilled surfactant to be adsorbed.

Ventilation of rats in vivo

The preparation techniques have been described previously [13, 14]. Briefly, animals were (re)anesthetized with 65% NO_2 /35% O_2 and 2% isoflurane. Subsequently, a sterile polyethylene catheter was inserted into a carotid artery for drawing arterial blood samples. Thereafter, a sterile metal cannula was inserted into the trachea (in all animals which had not received exogenous surfactant). After these surgical procedures, gaseous anesthesia was discontinued and anesthesia was continued with 60 mg/kg pentobarbital sodium i.p. (Nembutal; Algin, Maassluis, The Netherlands). Subsequently, muscle relaxation was induced by pancuronium bromide 2 mg/kg, i.m. (Pavulon; Organon Teknika, Boxtel, The Netherlands), and ventilation was initiated with a Servo Ventilator 300 (Siemens Elema, Solna, Sweden) in a pressure-constant time-cycled mode and an FiO_2 of 1.0.

Rats (n=4 per group), were mechanically ventilated with normal pressure (13/3; PIP/PEEP in cm H_2O) or with high pressure (45/10) for 30 or 120 min, leading to tidal volumes of 12 ml/kg and 18 ml/kg respectively [12]. At the end of the study period, heparinized blood was taken from the arterial line, and animals were then killed with an overdose of pentobarbital. The unventilated control group was killed immediately after tracheotomy in an identical way. The thorax was opened and lungs were collected sterile, snap frozen and stored.

The animals were randomly allocated into 3 main experimental groups: (1) Ventilation without any treatment, (2) NaCl-instillation prior to ventilation, (3) surfactant-instillation prior to ventilation.

Western blot analysis

Frozen lungs were powdered with a pestle in the constant presence of liquid nitrogen. Aliquots of the lung powder were lysed and homogenized in a buffer (50 mM Tris-Cl, pH 6.8, 150 mM NaCl, and 1% Triton X-100) containing Pefablock (1 mM), aprotinin (1µg/ml), pepstatin (1µg/ml), leupeptin (1µg/ml), NaF (1mM), Na_3VO_4 (1mM), ß-Glycerolphosphate (1mM). After 20 min on ice, the lysates were collected by pelleting the cellular debris for 15 min at 13,000 *g*. Total protein content was determined by a commercially available test (Pierce, Rockford, IL, USA).

An equal amount of protein (60µg/slot) was size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Dassel, Germany) and then immunoblotted with primary antibodies (P-Erk and P-p38, New England Biolabs (NEB), Frankfurt, Germany; P-Akt, Sigma, Deisenhofen, D) and horseradish peroxidase-conjugated secondary antibodies (NEB). Detection of the bound antibody with LumiGLO chemiluminescent substrate was performed according to the manufacturer (NEB). The densitometric analysis was performed with OPTIMAS 6.2 software (Optimas Corporation, Bothel, WA, USA).

Statistical analysis

The intensity of the bands in the blots was quantified and expressed as a ratio of a value from an unventilated control animal. The serum concentrations and the phosphorylation of AKT, ERK 1/2 and p38 were analysed by three-factorial ANOVA (SPSS 11.0, SPSS GmbH Software München) with the mode of ventilation (13/3 vs. 45/10 cm H_2O), the time of ventilation (30 min vs. 120 min) and surfactant pretreatment (yes or no) as the factors.

RESULTS

Oxygenation. The 120 min of ventilation with 45 cm H_2O/10 cm H_2O (PIP/PEEP) were well tolerated as indicated by the normal blood oxygenation (Table 1). In all animals, the arterial pO_2 was above 520 mm Hg at the end of the experiment. Because there never were significant differences between the untreated and the NaCl-injected animals, for this and the following analyses, these two groups were pooled together.

Table 1: Arterial oxygenation in mmHg

	13/3 cm H_2O PIP/PEEP		45/10 cm H_2O PIP/PEEP	
	30 min	120 min	30 min	120 min
Control	557±42 (11)	553±40 (8)	613±45 (9)	540±88 (7)
Surfactant	540±28 (8)	546±44 (4)	592±41 (8)	552±18 (4)

in parenthesis number of animals

TNF-α. In contrast to the other two cytokines (see below), serum TNF-α levels were not increased by alveolar overdistension alone (P = 0.351) (Fig. 1). This is consistent with previous findings of Verbrugge *et al.* in surfactant-depleted and non-depleted rats [14]. However, in the presence of surfactant (P = 0.045) a significant increase of TNF-α over time

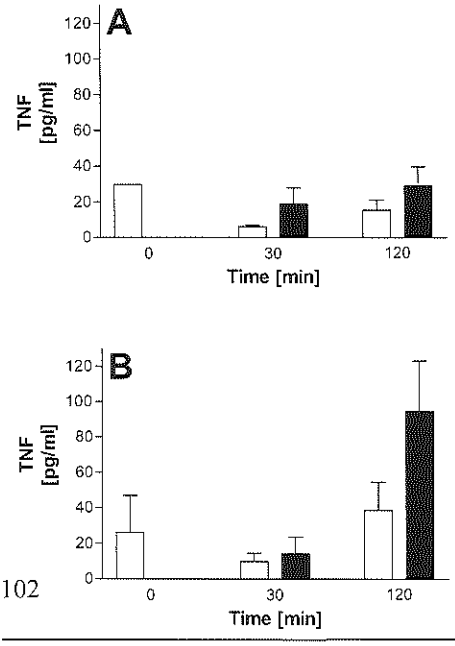

Figure 1
Effect of ventilation strategy, time and surfactant on serum levels of TNF. Rats were either left non-ventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3, PIP/PEEP in cm H_2O, Panel **A**) or with high pressure (45/10, Panel **B**) for 30 or 120 min. Open bars, non-treated animals or animals treated with NaCl. Solid bars, animals treated with surfactant. TNF serum levels were measured by rat specific ELISA. Data are shown as the mean ± SEM from 4 to 7 animals. The statistical analysis is given in the text.

($P = 0.006$) was observed (Fig. 1).

IL-6. There was no effect of supra-physiological surfactant levels on mediator release ($P = 0.244$), but there was a strong increase in IL-6 over time ($P = 0.002$) induced by ventilation with 45/10 cm H_2O ($P = 0.0004$) (Fig. 2). Thus, overdistension increased IL-6 serum levels *in vivo*, and the concentrations were higher after 120 min than after 30 min.

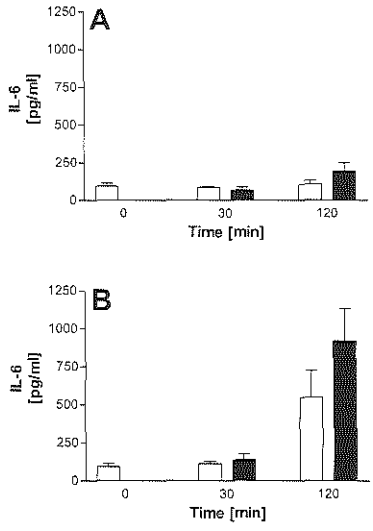

Figure 2
Effect of ventilation strategy, time and surfactant on serum levels of IL-6. Rats were either left non-ventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3, PIP/PEEP in cm H_2O, Panel **A**) or with high pressure (45/10, Panel **B**) for 30 or 120 min. Open bars, non-treated animals or animals treated with NaCl. Solid bars, animals treated with surfactant. IL-6 serum levels were measured by rat specific ELISA. Data are shown as the mean ± SEM from 4 to 7 animals. The statistical analysis is given in the text.

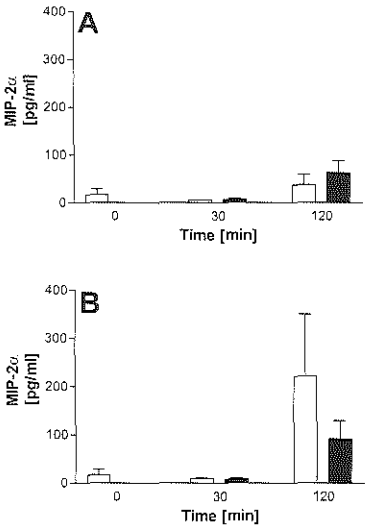

Figure 3
Effect of ventilation strategy, time and surfactant on serum levels of MIP-2α Rats were either left non-ventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3, PIP/PEEP in cm H_2O, Panel **A**) or with high pressure (45/10, Panel **B**) for 30 or 120 min. Open bars, non-treated animals or animals treated with NaCl. Solid bars, animals treated with surfactant. MIP-2α serum levels were measured by rat specific ELISA. Data are shown as the mean ± SEM from 4 to 7 animals. The statistical analysis is given in the text.

MIP-2α. Again, there was a strong increase of MIP-2α serum concentrations over time ($P < 0.0001$), and a clear effect of the mode of ventilation ($P = 0.022$) (Fig. 3). These responses were not affected by surfactant ($P = 0.237$).

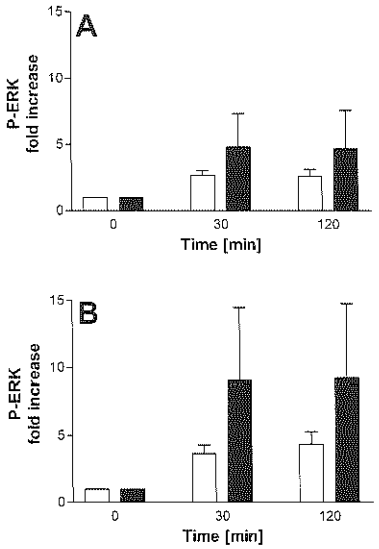

Figure 4
Effect of ventilation strategy, time and surfactant on the activation of ERK. Rats were either left non-ventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3, PIP/PEEP in cm H_2O, Panel **A**) or with high pressure (45/10, Panel **B**) for 30 or 120 min. Open bars, non-treated animals or animals treated with NaCl. Solid bars, animals treated with surfactant. P-ERK was analysed by immunoblot, using antibodies specific for the phosphorylated form. The densitomteric data from p42 (ERK1) and p44 (ERK2) were averaged and are shown as fold increase over the unventilated control and represent the mean ± SEM from 4 to 7 animals. The statistical analysis is given in the text.

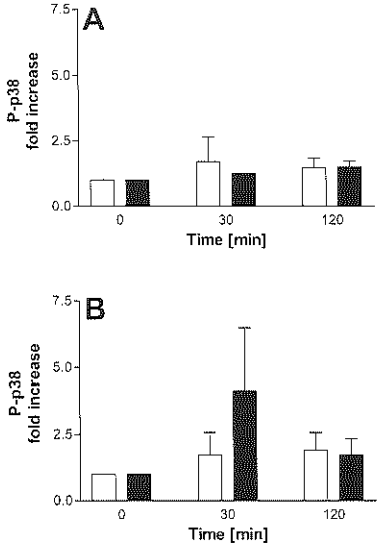

Figure 5
Effect of ventilation strategy, time and surfactant on activation of p38 kinase. Rats were either left non-ventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3, PIP/PEEP in cm H_2O, Panel **A**) or with high pressure (45/10, Panel **B**) for 30 or 120 min. Open bars, non-treated animals or animals treated with NaCl. Solid bars, animals treated with surfactant. P-p38 was analysed by immunoblot, using antibodies specific for the phosphorylated form. The densitomteric data are shown as fold increase over the unventilated control and represent the mean ± SEM from 4 to 7 animals. The statistical analysis is given in the text.

ERK. We have previously shown that ventilation with 45/10 cm H_2O activates ERK in alveolar type II epithelial cells [6]. This was confirmed in the present series of experiments

(Fig. 4), where ventilation with higher pressures clearly increased phosphorylation of ERK1/2 ($P = 0.009$). These responses were not affected by surfactant ($P = 0.377$). There was no difference in ERK activation between 30 min or 120 min ventilation ($P = 0.892$). Thus, the ventilation-induced activation of ERK is complete at latest after 30 min, lasts for at least two hours and is not influenced by exogenous surfactant.

P38 kinase. As noted before [6], phosphorylation of p38 was not affected by the ventilation mode ($P > 0.05$). There was no effect of surfactant ($P > 0.05$) and even after 120 min there was no increase in P-p38 ($P > 0.05$) (Fig. 5).

AKT kinase. Ventilation with 45/10 cm H_2O led to activation of AKT kinase ($P = 0.037$), but there was no effect of prolonged overdistension ($P = 0.413$) or time ($P = 0.189$) (Fig. 6). Thus, similar to ERK, the ventilation-induced activation of AKT kinase is complete after 30 min, lasts for at least two hours and is not influenced by surfactant.

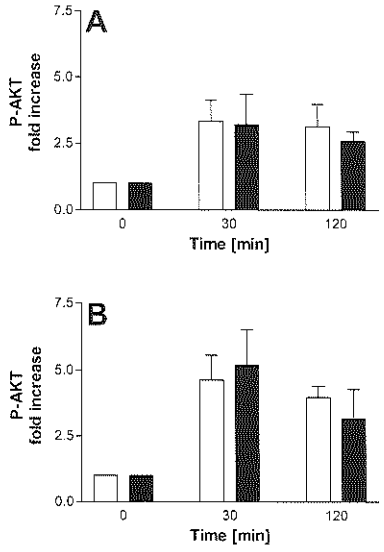

Figure 6
Effect of ventilation strategy, time and surfactant on activation of AKT kinase. Rats were either left non-ventilated (control, time point 0), or were mechanically ventilated with normal pressure (13/3, PIP/PEEP in cm H_2O, Panel **A**) or with high pressure (45/10, Panel **B**) for 30 or 120 min. Open bars, non-treated animals or animals treated with NaCl. Solid bars, animals treated with surfactant. P-AKT was analysed by immunoblot, using antibodies specific for the phosphorylated form. The densitomteric data are shown as fold increase over the unventilated control and represent the mean ± SEM from 4 to 7 animals. The statistical analysis is given in the text.

DISCUSSION

This study demonstrates that prolonged overdistension of a lung leads to the protracted activation of various signaling processes and to the release of pro-inflammatory mediators *in vivo*. All experiments were performed in healthy animals, where any increase in 'avleolar shear forces' by collapsed alveoli (atelectotrauma) was minimized by application of high

PEEP levels (10 cm H_2O), and in which a supplementary amount of exogenous surfactant was instilled to observe the effect of prolonged overstretching on ventilation-induced mediator release. Because an excessive amount of surfactant maintains the distensibility of lungs subjected to high distending forces, exogenous surfactant will conserve the stretch on the lung tissue exerted by mechanical ventilation [15]. Therefore, the current findings support the notion that the ventilation-induced signalling responses are dependent on stretching of alveolar units.

Exogenous surfactant as a therapy for acute lung injury is suggested to restore the amount of active surfactant in the injured lung and thus to help restore normal lung functions. Because patients with acute lung injury depend on mechanical ventilation, the interaction between ventilation-induced signaling and mediator release on the one hand and surfactant on the other is of great clinical interest. The present study essentially confirms previous data from isolated lungs [10, 16] in intact animals, demonstrating that ventilation-induced mediator release is either maintained or even heightened by exogenous surfactant.

Because of the intact chest wall, lungs cannot expand to the same degree *in vivo* as in isolated lungs [2]. Thus, it seems likely that overstretching occurs to a greater extent in isolated lungs than in healthy lungs *in vivo* [2]. In support of this notion, ventilation-induced release of TNF was noted in isolated lungs [17-19], but not *in vivo* [14]. This observation was confirmed in the present study, where ventilation with high pressures alone failed to raise serum TNF levels. However, we demonstrated that extending the overstretching potential of the lung by pretreatment with exogenous surfactant, combined with high inspiratory pressures, results in elevated serum TNF levels. These data suggests that exogenous surfactant when instilled into healthy lungs (which will never be the case in humans), might increase the risk of biotrauma when these lungs are subsequently subjected to overinflation. Notably, overventilation alone was sufficient to elevate serum levels of IL-6 and MIP-2α. These findings are in line with previous studies in isolated mouse lungs, where IL-6 and MIP-2α were released in much higher quantities than TNF [3].

It is highly unlikely that the increased TNF production in surfactant-treated lungs was a result of direct immunostimulating properties of our surfactant preparation. Surfactant was given intratracheally while the mediators were assessed systemically, there was no effect of surfactant on mediator release in the 13/3 groups and in addition, we have shown that compartmentalization is maintained in rats ventilated with 45/10 cm H_2O PIP/PEEP [13].

Moreover, other studies have shown that the immuno-modulating properties of surfactant treatment reduce rather than increase mediator release [20-23]. Therefore, our current findings suggest that exogenous surfactant acted by facilitating overdistension rather than by direct stimulation of TNF producing cells.

The present data extend our previous findings on the ventilation-induced activation of ERK, in which we examined ERK activation for only 60 min [6]. The present study shows that this activation lasts for at least 120 min. This is in contrast to studies with freshly isolated and alveolar epithelial cells, in which the stretch-induced ERK activation lasted for only 30 min [5]. The ventilation-induced activation of ERK tended to be higher in surfactant-treated rats, but this effect did not reach significance level. Because prolonged activation of ERK may promote proliferation [24], in future studies it will be important to address the long-term activation of ERK during ventilation.

A novel observation in this study was the activation of AKT kinase by ventilation with high pressure, a response that was not influenced by exogenous surfactant. Because it is known that shear stress may activate AKT kinase [8] and because ventilation with increased positive pressure will increase pulmonary artery pressure and thus vascular shear stress [25], in the present experiments we cannot exclude vascular shear stress as a stimulus for the enhanced AKT-phosphorylation in response to ventilation with high distending pressures. However, in experiments with isolated perfused mouse lungs application of high negative pressure (a condition that decreases shear stress) also led to elevated activation of AKT in capillary endothelial cells [26], suggesting that both stretch and vascular shear stress might contribute to the activation of AKT kinase *in vivo*. The consequences of AKT-kinase activation during ventilation are unknown, but from the literature it is known that this might lead to decreased apoptosis [27] and/or to increased NO production through phosphorylation of endothelial NO synthase [8].

In summary, we have shown that prolonged ventilation with high pressure leads to elevated serum levels of MIP-2α and IL-6 as well as to activation of AKT-kinase and ERK 1/2 *in vivo*. Exogenous surfactant did not affect any of these responses, and enhanced ventilation-dependent TNF-α serum levels, probably by allowing for a longer period of stretch. These findings suggest that alveolar overdistension, also during application of exogenous surfactant, can result in mediator release in patients with acute lung injury and that ventilation settings should be optimized for the individual lung [28].

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Chapter 7

Ventilator-induced heat shock protein 70 and cytokine mRNA expression in an endotoxin challenged rat lung

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submitted

ABSTRACT

Objective: To investigate the effect of different mechanical ventilation strategies on heat shock protein 70 and pulmonary inflammatory cytokine expression in a moderately inflamed lung.

Study design and setting: Prospective, randomized, experimental animal study.

Subjects and interventions: 42 male Sprague-Dawley rats were challenged intratracheally with endotoxin. After 24 hours the rats were randomly assigned to one of the ventilation strategies. Either rats received 4 hours of mechanical ventilation without PEEP, or mechanical ventilation with PEEP. A non-ventilated control group did only receive LPS. Heat shock protein 70 and cytokine mRNA levels in the lung were measured. Lung pathology after LPS challenge was evaluated by histology.

Main results and conclusions: PaO_2 levels and lung histology revealed no deterioration after PEEP ventilation and severe deterioration after ZEEP ventilation. There was a significant increase in the expression of HSP70 and IL-1β mRNA in the lungs of the ZEEP group compared to the PEEP group and non-ventilated controls. TNF-α and IL-6 showed a similar trend; in the ZEEP group, high HSP70 levels correlated inversely with low IL-1β and IL-6 mRNA. We propose that HSP70 expression is a protective response of the lung against ventilator-induced lung injury via a decrease in cytokine transcription.

INTRODUCTION

Mechanical ventilation is an important life saving procedure that may, however, induce or aggravate lung injury [1]. Ventilator-induced lung injury (VILI) in animal models is characterized by permeability pulmonary edema with protein-rich alveolar fluid and may be prevented by the use of positive end-expiratory pressure (PEEP) [2-5]. Overstretch of lung tissue leading to mechanical disruption and surfactant inactivation have been proposed as underlying mechanisms of VILI. However, a ventilator-induced inflammatory response, characterized by cytokine release in the lung is possibly a contributing mechanism [6-8]. Moreover, locally produced ventilator-induced inflammatory mediators might spill over from the lung into the circulation and thus generate distal organ failure [9-12].

A number of experimental studies support the hypothesis that intermittent stretch of lung tissue during mechanical ventilation induces inflammatory cytokines [6-8, 13-15]. *In vitro* stretching of unstimulated alveolar epithelial cells causes the production of IL-8 [13]. Repetitive stretching of lipopolysaccharide (LPS) stimulated alveolar macrophages *in vitro* leads to the production of the cytokine IL-8, as well as of TNF-α and IL-6 [14]. However, data on the production and release of inflammatory mediators after mechanical ventilation of healthy lungs are conflicting [7, 15]. Ricard et al. found no cytokine release in normal rat lungs after *ex vivo and in vivo* ventilation [15], whereas Tremblay et al. found an increase in cytokine production in the *ex vivo* ventilated lung [7]. Both groups agree that pre-existing inflammation before mechanical ventilation can lead to cytokine release in the lung. In surfactant-depleted rats, induction of VILI by high volume and low PEEP ventilation did not lead to an increase in TNF-α plasma levels or in broncho-alveolar lavage fluid (BAL) [16]. Also the results of studies in humans as to ventilator-induced inflammatory response are conflicting. In ARDS patients, higher tidal volumes and lower PEEP increased intra-alveolar concentrations of inflammatory mediators [11]. Wrigge et al. did not find an inflammatory response in adults with healthy lungs, ventilated during surgery [17]. In contrast, we have recently found pro-inflammatory changes in the tracheal aspirate and blood of children with normal lungs ventilated for only 2 hours during heart catheterization [18].

Heat shock proteins (HSPs) are produced as a response of cells to stress and are synthesized after exposure to various harmful stimuli (heat, hypoxia, endotoxins cytokines and oxygen radicals) [19, 20]. It seems reasonable to assume that stressing lung tissue by mechanical

ventilation would also induce heat shock proteins. The HSP70 family, the most prominent class of HSPs, contains two major members, a 73-kDa constitutive form and a 72-kDa inducible form. The inducible form (so-called HSP70) is located in the nucleus and cytosol of the cell and is essential for cell survival under stressful conditions [19-23]. HSPs are molecular chaperones, which are involved in maintaining the conformational and structural integrity of intracellular proteins. In addition, HSPs are capable of reducing inflammation, via the inhibition of cytokine production [21, 22, 24-26]. HSP70 expression in the lung may be regarded as marker for pulmonary stress [27], but is also associated with a decrease in mortality after acute lung injury [28]. There seems to be a direct association between HSP expression and cytokine production. *In vitro* HSP induction in respiratory epithelial cells decreases the expression of cytokines by preventing NF-κB activation [29]. The present study was designed to investigate the effect of mechanical ventilation on the expression of HSP70 and cytokine mRNA in a moderately inflamed lung. Our hypothesis was that ZEEP ventilation would increase the inflammatory response in the lung and that PEEP ventilation would attenuate this response.

To answer the question whether HSP70 is involved in the downregulation of cytokine mRNA expression, we determined the correlation between HSP70 and cytokine mRNA levels in lung tissue.

METHODS

Animals

A total of 42 Sprague-Dawley rats (250-300 g) were obtained from Harlan CPB (Zeist, The Netherlands). All animals were handled in accordance with European Community Guidelines. In 38 animals lung injury was induced by intratracheally aerosolization of 16mg/kg of *Salmonella enteritidis* (Sigma L6761) lipopolysaccharide (LPS), under gaseous anesthesia, 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane; Pharmachemie BV, Haarlem, The Netherlands), using a miniature nebulizer (Penn-Century, Philadelphia, PA, USA) [30]. After 24 hours, 28 rats were re-anesthetized as described above and tracheotomized. A sterile metal cannula was inserted into the trachea and a polyethylene catheter was inserted into the carotid artery. Subsequently, gaseous anesthesia was discontinued and anesthesia was continued with 60 mg/kg/h pentobarbital sodium (Nembutal;

Algin BV, Maassluis, The Netherlands) given intraperitoneally. Muscle relaxation was attained with 2 mg/kg/h pancuronium bromide (Pavulon; Organon Technika, Boxtel, The Netherlands) intramuscularly. After muscle relaxation the animals were connected to a ventilator (Servo Ventilator 300; Siemens-Elema, Solna, Sweden). Blood gas determinations were performed every hour, using a pH/blood gas analyzer (ABL 505, Radiometer, Copenhagen, Denmark). Body temperature was maintained at 37°C with heating lamps.

Experimental protocol

Rats were randomized to the following ventilation strategies: 1) ZEEP group: Peak inspiratory pressure (PIP)=18 cmH_2O; PEEP=0 cmH_2O (n=14) and 2) PEEP group: PIP=18 cmH_2O; PEEP=4 cmH_2O (n=14). Rats were ventilated during 4 hours in a pressure-controlled time-cycled mode, at a fractional inspired oxygen concentration (FiO_2) of 1.0, I/E ratio of 1:2 and a frequency between 20-30/minute to maintain normocapnia. The ventilated animals were sacrificed with an overdose of intra-arterial administered pentobarbital sodium. 6 rats received LPS only and served as non-ventilated controls (NVC). The non-ventilated controls were sacrificed under gaseous anesthesia after 28 hours.

Lung histology

To evaluate the induction of lung injury by intratracheally LPS aerosolization, lungs of 8 rats were removed after 24h. 4 rats received LPS intratracheally and 4 rats PBS. For fixation 4% paraformaldehyde was instilled in the lungs. Histology was assessed by hematoxylin and eosin staining. The lungs of 8 ventilated rats (4 ZEEP, 4 PEEP) were snap-frozen after tissue-tek instillation. The presence of HSP70 in the lung was evaluated by standard immunohistochemistry procedures using a mouse monoclonal antibody against HSP70 (Stressgen Biotechnologies, Victoria, Canada). Primary antibody binding was visualised with horse radish peroxidase conjugate and diaminobenzidine.

Western blotting

Total lung homogenates of 26 rats were prepared from frozen whole lungs (-80°C). 50 μg of protein was separated by polyacrylamide gel electrophoresis. Expression of HSP70 and IκBα proteins was determined by Western blotting. Proteins were labeled with a mouse monoclonal antibody against HSP70 (Stressgen Biotechnologies, Victoria, Canada) and a rabbit polyclonal against IκBα (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed using horseradish peroxidase-linked antibodies and chemiluminescence (ECL) by autoradiography.

Cytokines

Apart from protein, mRNA was isolated from part of the total lung homogenates (ZEEP n=6, PEEP n=4, NVC n=6). For determination of cytokine mRNA, the RNase Protection kit and a multiprobe template set for rat cytokines IL-1β, TNF-α and IL-6 (Pharmingen, San Diego, CA, USA) were used according to the manufacturer's instructions. mRNA expression was analyzed by phosporimaging, quantitated using the software Molecular Analyst, version 1.5 (Biorad, Richmond, CA, USA) and expressed as percentage of housekeeping gene expression. TNF-α was measured by ELISA (U-cytech, Utrecht, The Netherlands) according to the manufacturer's protocol.

Statistics

Values are expressed as mean ± standard error of the mean. Bodyweight was analyzed by Student's *t*-test and blood gas analysis by two-way analysis of variance (ANOVA). All other parameters were analyzed by one-way analysis of variance (ANOVA) and Newman-Keuls's post-test. Correlations were calculated by Pearson's analysis. A P-value <0.05 was considered as statistically significant.

RESULTS

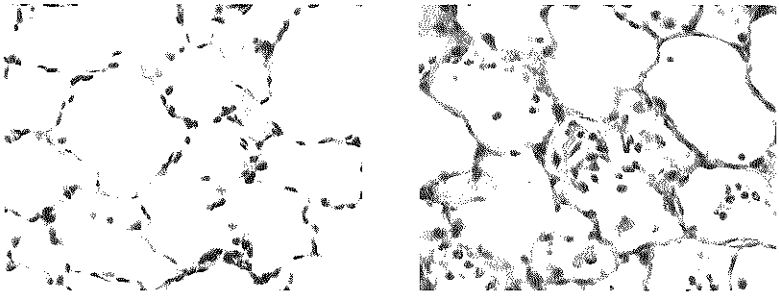

Figure 1
The lung injury model. Representative hematoxylin and eosin stained paraffin sections of rat lung tissue.
A) Lung tissue 24 h after PBS aerosolization; B) Lung injury 24 hours after LPS aerosolization. Lung histology is characterized by marginalization of polymorphonuclear leukocytes (PMNs) in the blood vessels and exudation of PMNs into the alveolar space. Original magnification 40x.

Lung injury model

To induce lung injury LPS was aerosolized intratracheally. After 24 hours rats developed respiratory distress, with tachypnea, raised fur and significant weight loss (from 284 ± 3.6 g to 259 ± 3.3 g 24 h later, $p<0.001$). Lung injury was characterized by marginalization of polymorphonuclear leukocytes (PMNs) in the blood vessels and exudation of PMNs towards the alveolar space, sometimes accompanied by fibrin strands (figure 1).

Gas exchange

Two different ventilatory strategies were applied to the rats with moderately inflamed lungs 24 hours after LPS aerosolization. We studied the effect of 4 hours ZEEP (18/0) and PEEP (18/4) ventilation on oxygenation levels. In the ZEEP group, PaO_2 levels deteriorated significantly over time from 585 ± 32.9 mm Hg at t=0 hours to 262 ± 65.0 mm Hg at t=4 hours ($p<0.05$). In the PEEP group, PaO_2 levels remained stable, with values of 514 ± 32.9 at t=0 hours and 511 ± 23.0 at t=4 hours.

$PaCO_2$ levels did not change during the experiment: total levels in the ZEEP and PEEP group were 30 mm Hg ± 2.3 at t=0 hours and 38 ± 4 mm Hg at t=4 hours.

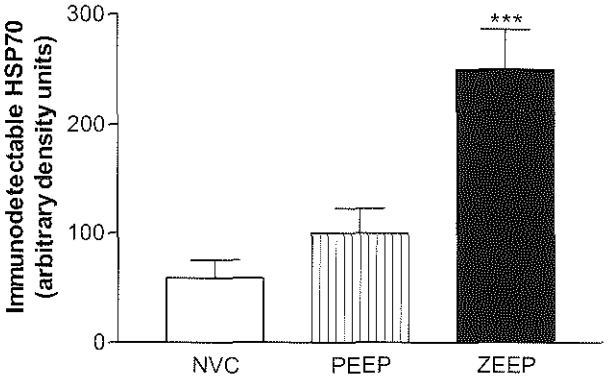

Figure 2
HSP70 expression in the lung. Effect of two different ventilation strategies on HSP70 levels in a moderately inflamed rat lung. HSP70 expression in 50 µg protein from total lung homogenates was analyzed by Western blotting. Data represent arbitrary density units. ZEEP group (n=10), PEEP group (n=10) and non-ventilated control group (NVC) (n=6). (***$p<0.001$ compared with the other groups)

HSP 70 expression in the lung

The effect of the two different ventilation strategies on HSP70 expression in moderately inflamed lung tissue was measured after 4 hours of mechanical ventilation. Western blot analysis showed that HSP70 expression in the ZEEP group (249.0±36.6) was significantly higher than in the non-ventilated control group (NVC) group (58.8±16.2) ($p<0.001$) and in the PEEP group (99.9±23.0) ($p<0.001$). HSP70 levels in the PEEP group were not significantly different from HSP70 levels in the NVC group (figure 2). Immunohistochemistry of HSP70 on representative frozen lung sections was consistent with Western blot analysis. In the ZEEP group HSP70 immunoreactivity was mainly observed in the respiratory epithelial cells and interstitial macrophages of the lung. This was in contrast to the PEEP group where only a few macrophages were stained positive. Overall, the ZEEP lung showed severe deterioration with atelectasis and consolidaton of lung tissue (figure 3).

Figure 3
HSP70 immunohistochemistry. Immunohistochemical analysis of HSP70 in representative frozen rat lung sections after 4 hours of mechanical ventilation. A) PEEP ventilated (18/4) lungs; B) ZEEP (18/0) ventilated lungs. In the ZEEP group HSP70 immunoreactivity was mainly observed in the respiratory epithelial cells and interstitial macrophages of the lung. The PEEP ventilated lung showed staining in a few macrophages only. Overall, the ZEEP lung showed severe deterioration with atelectasis and consolidaton of rat lung tissue.

Expression of cytokine mRNA in the lung

To study the effect of mechanical ventilation on cytokine mRNA expression, we analyzed the expression of IL-1β, TNF-α and IL-6 mRNA in lungs. Rats were treated with LPS and the 4-hour mechanical ventilation period started 24 hours later. The expression of IL-1β mRNA was significantly higher in the ZEEP (18/0) group than in the PEEP ventilated group (18/4) ($p<0.01$). IL-1β mRNA in the PEEP group did not differ significantly from the expression in the control group (NVC). The expression of IL-6 and TNF-α mRNA showed a similar trend as IL-1β mRNA, but did not reach statistical significance (figure 4).

To investigate whether there is a relation between cytokine and HSP70 levels, correlations between HSP70 expression and IL1-β, Il-6 and TNF-α mRNA expression were calculated. In the ZEEP group, IL-1β and IL-6 mRNA correlated negatively with HSP70 levels ($p= 0.045$, $r= -0.82$ and $p=0.01$, $r= -0.9$, respectively). The correlation between TNF-α mRNA and HSP70 showed the same tendency ($p=0.12$, $r= -0.7$) (figure 5). No such correlations were found in the PEEP group and the non-ventilated control group.

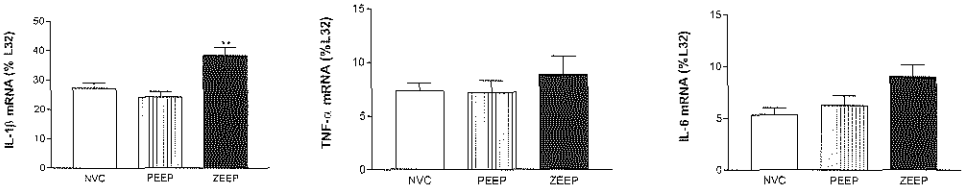

Figure 4
Cytokine expression in the lung. Effect of 4 hours mechanical ventilation on the expression of IL-1β (panel A), IL-6 (panel B) and TNF-α (panel C) mRNA, expressed as percentage of the household gene (L32) expression. ZEEP group (n=6), PEEP group (n=4) and non-ventilated control group (NVC) (n=6). (* $p<0.01$).

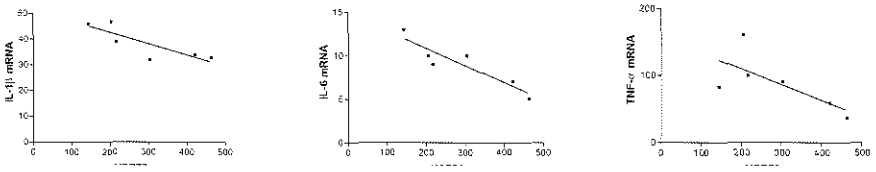

Figure 5
Correlation between effect of 4 hours mechanical ventilation on HSP70 and cytokine mRNA expression in the ZEEP ventilated group. IL-1β: $p= 0.045$, $r= -0.82$; IL-6: $p=0.01$, $r= -0.9$; TNF-α, $p=0.12$, $r= -0.7$.

The ventilation strategies used in this study did not induce detectable levels of TNF-α protein in total lung homogenates (<15pg/mg protein) or in plasma (<15pg/ml). Moreover, we did not detect TNF-α protein in lung homogenates or plasma from the non-ventilated control animals.

Correlation of IκBα andHSP70

The transcription of mRNA for pro-inflammatory cytokines is dependent on the activation of the transcription factor NF-κB, a process that involves the degradation of the inhibitory protein IκBα. It has been proposed that HSP70 is capable of inhibiting IκBα degradation. In view of the inverse correlation between HSP70 levels and cytokines mRNA, we analyzed the correlation between IκBα and HSP70 expression. There were no significant differences between the protein levels of IκBα in the ZEEP (97.1±9.8), PEEP (99.0±4.6) and NVC group (87.8 ±7.2). However, the level of IκBα correlated positively to the HSP70 expression level ($p<0.01$ r 0.77) in the ZEEP group. In the PEEP group and NVC group no correlations between HSP70 and IκBα levels were found (figure 6).

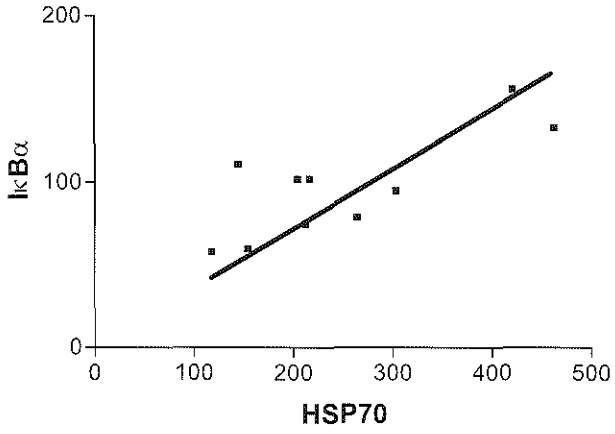

Figure 6
HSP70 and IκBα expression in ZEEP ventilated lungs were determined by Western blotting and Pearson correlations were calculated. $p<0.01$, $r = 0.77$.

DISCUSSION

The present study investigated the effect of mechanical ventilation in a moderately inflamed lung, in the absence and presence of PEEP, on the expression of HSP70 and pro-inflammatory cytokines in the rat lung. After 4 hours of mechanical ventilation, HSP70 expression in the ZEEP group increased significantly and showed a positive correlation with IκBα levels. In the ZEEP group IL-1β mRNA expression was increased and the same trend was seen for IL-6 and TNF-α mRNA expression. HSP70 levels in this ZEEP group, showed negative correlations with IL-1β and IL-6 mRNA and tended to correlate negatively with TNF-α mRNA expression.

Our findings are in line with the concept that mechanical ventilation can induce or increase an inflammatory response in the lung. Ventilator-induced lung inflammation, called biotrauma, was first described by Tremblay et al [7]. They demonstrated that *ex vivo* ventilation with high volume and zero PEEP leads to induction and increase of IL-1β, MIP-2, IL-10 and TNF-α in the lung, irrespective of prior LPS treatment. Von Bethmann et al. demonstrated that high volume ventilation could induce the release of cytokines in healthy perfused mouse lungs [8]. Thus, *ex vivo* ventilation of healthy rodent lungs can induce increased pro-inflammatory cytokine expression. However, in intact animals there is less evidence that ventilation as such is sufficient to induce cytokine production. In intact healthy rats Ricard et al. could not find an inflammatory response after mechanical ventilation [15]. Even in a model of severe lung injury induced by lavage, 4 hours of mechanical ventilation of intact animals did not result in an increase in TNF-α in the BAL and serum [16]. In addition, Chiumello et al. only found differences in lung fluid TNF-α and MIP-2 between different ventilation strategies after 4 hours of ventilation in HCl treated rats, if corrected for lung fluid concentrations [10]. In our experiments we have shown that *in vivo* ventilation of rat lungs after intratracheal exposure to LPS does induce increased expression of mRNA for proinflammatory cytokines. Apparently, subtle differences in ventilatory strategy can have important consequences for pro-inflammatory cytokine expression, exacerbation of the lung pathology and PaO_2 levels.

To our knowledge, an effect of mechanical ventilation per se on HSP70 expression in the lung has not been described before. There is evidence that mechanical stretch in isolated rabbit and rat hearts can lead to HSP70 expression [31, 32]. HSPs protect cells after injurious

stimuli, by refolding and stabilization of denatured protein aggregates and by transcriptional inhibition of cytokine production [15, 33, 34]. Heat shock, Ca^{2+} increasing agents, cAMP and PKC stimulators, ischemia, sodium arsenite, microbial infections, nitric oxide, hormones and TNF-α can all increase or induce HSPs [35-41]. Recently, it has been shown that *ex vivo* mechanical ventilation in lungs from pre-heated animals, results in elevated HSP70 levels in lung tissue and lower BAL cytokine levels of TNF-α, IL-1β and MIP-2 [42]. However, the injurious ventilation strategy itself (Vt 40 ml/kg, PEEP 0) did not induce or further enhance HSP70. In our study, we have shown that injurious ventilation of moderately inflamed lungs can induce an increase in HSP70 expression. There are two important differences between the two studies: first, we have ventilated rat lungs *in vivo* and, second, we have used a model of moderate lung inflammation. In the ZEEP group we have found a negative correlation between HSP70 protein expression and the expression of pro-inflammatory cytokines mRNA. This negative correlation supports the concept that HSP70 has an anti-inflammatory function. HSPs may prevent the transcription of pro-inflammatory cytokines by inhibition of IκBα degradation. This degradation is necessary for NF-κB activation and induces transcription of most pro-inflammatory cytokine genes, such as IL-1β, TNF-α and IL-6. *In vitro* experiments in lung epithelial cells revealed that HSP70 is able to block IκBα degradation, possibly through the inhibition of IκB kinase (IKK) activation [20]. In our study the expression of HSP70 expression was positively correlated with the level of the inhibitory protein IκBα in the ZEEP ventilated group. However, in the PEEP ventilated group and the non-ventilated control group no correlations were found. The fact that only the high HSP70 levels in the ZEEP group correlate with cytokine mRNA and IκBα levels, suggests that the HSP70 protein needs to exceed a certain threshold level before HSP is able to decrease cytokine transcription. In conclusion, an increase in HSP70 in the lung can be regarded as an endogenous protective mechanism against ventilator-induced lung injury.

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Chapter 8

Application of the open lung concept during positive pressure ventilation reduces pulmonary inflammation in newborn piglets

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ABSTRACT

It has been shown that application of the open lung concept (OLC) during high-frequency oscillatory ventilation (HFOV) attenuates pulmonary inflammation. We hypothesized that this attenuation could also be achieved by applying the OLC during positive pressure ventilation (PPV). After repeated whole lung-lavage newborn piglets were assigned to one of three ventilation groups: 1) PPV_{OLC}; 2) $HFOV_{OLC}$ or 3) conventional PPV (PPV_{CON}). After a ventilation period of 5 h, analysis of broncho-alveolar lavage fluid showed a reduced influx of polymorphonuclear neutrophils, interleukin-8 and thrombin activity in both OLC groups compared to the PPV_{CON} group. There were no differences in tumor necrosis factor alpha levels. We conclude that application of the OLC during PPV reduces pulmonary inflammation compared to conventional PPV and the magnitude of this reduction is comparable to HFOV.

INTRODUCTION

Acute respiratory failure in newborns is often treated with mechanical ventilation. Although mechanical ventilation optimizes gas exchange, it may also induce secondary lung damage often referred to as ventilator-induced lung injury [1]. If this secondary lung damage is not resolved or abnormally repaired, it may lead to a chronic pulmonary disorder often called chronic lung disease of the newborn (CLD) [2].

It has been suggested that pulmonary inflammation plays an important role in the process of CLD [2, 3]. Indeed, several studies examining the broncho-alveolar lavage (BAL) fluid from newborns ventilated for respiratory distress syndrome (RDS), showed increased influx of polymorphonuclear neutrophils (PMN) [4, 5] and increased levels of inflammatory mediators like interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α) in patients developing CLD [6-10]. Furthermore, there is increasing evidence that besides its critical role in hemostasis, thrombin is also involved in the process of pulmonary inflammation and fibrosis [11].

Although several risk factors have been postulated for this increase in inflammation, injurious mechanical ventilation is considered of major importance [1, 2]. This has led to the assumption that lung protective ventilation strategies could attenuate the inflammatory response in the lung during mechanical ventilation. Based on previous studies evaluating the damaging aspects of mechanical ventilation, these protective strategies should include optimal alveolar recruitment, prevention of alveolar collapse during expiration and prevention of alveolar overdistension during inspiration [12-14]. The ventilation strategy based on these three important principles of lung protective ventilation is also referred to as the "Open Lung Concept" (OLC) [15].

Up to now only studies comparing conventional positive pressure ventilation (PPV_{CON}) to high-frequency oscillatory ventilation using the OLC ($HFOV_{OLC}$), have shown a reduction in pulmonary inflammation in favor of the OLC [16, 17].

Recently two studies in adult animals demonstrated that applying the OLC during positive pressure ventilation (PPV_{OLC}) is also feasible and resulted in superior gas exchange and a reduction of several non-inflammatory parameters of ventilator-induced lung injury comparable to $HFOV_{OLC}$ [18, 19]. To date no previous studies investigated the inflammatory changes during PPV_{OLC} in surfactant-depleted newborn animals. We therefore decided to explore the possible inflammatory changes during PPV_{OLC} in surfactant-depleted newborn

piglets and compared these changes to $HFOV_{OLC}$ and PPV_{CON} because these latter two strategies are mostly used in neonates. Inflammation was assessed by alveolar PMN influx, levels of IL-8, TNF-α
and thrombin activity in BAL fluid. We hypothesized that PPV_{OLC} would lead to less pulmonary inflammation as compared to PPV_{CON} and that the magnitude of this reduction would be comparable to $HFOV_{OLC}$.

MATERIAL AND METHODS

All experiments were performed at the Department of Anesthesiology, Erasmus University Rotterdam. The study was approved by the institutional Animal Investigation Committee and the care and handling of the animals were in accord with European Community guidelines.

Animal preparation

Anesthesia was induced in 24 newborn piglets, aged 25 ± 13 (SD) h and weighing 1.5 ± 0.3 (SD) kg, with ketamine hydrochloride (35 mg/kg, i.m.) and midazolam (0.5 mg/kg, i.m.). The animals were tracheotomized, connected to a Servo ventilator 300 (Siemens-Elema, Solna, Sweden) and ventilated in the pressure controlled time-cycled mode, at a fractional inspired oxygen concentration (FiO_2) of 1.0, rate of 25-30 breaths per minute, peak inspiratory pressure (PIP) of 10-12 cmH_2O, positive end-expiratory pressure (PEEP) of 2 cmH_2O and an inspiratory/expiratory (I/E) ratio of 1:2. A neuromuscular block was induced with pancuronium bromide (0.5 mg/kg, i.v.) and anesthesia was maintained with a continuous infusion of fentanyl (20 μg/kg/h), midazolam (0.3 mg/kg/h) and pancuronium bromide (0.3 mg/kg/h).

A 4-Fr double lumen polyurethane catheter (Vygon, Ecouen, France) was introduced through the external jugular vein for infusion of fluids and medication. The carotid artery was canulated for monitoring of blood pressure and blood sampling. In addition, a sensor for continuous blood gas monitoring (Paratrend/ Trendcare, Philips Medical, Böblingen, Germany) was inserted through a femoral artery catheter.

A continuous infusion of 5% dextrose was started (100 ml/kg/d) and all animals received one dose of cefotaxim (100 mg/kg). Body temperature was kept between 38° and 39°C during the experiment.

Surfactant depletion

After the instrumentation period respiratory failure was induced by repeated saline lavage (50 ml/kg; 37 °C) as described by Lachmann et al. [20]. The first 5 lavages were performed in the prone position after which the animals were placed in a supine position for the remainder of the lavage procedure. Lavages were repeated at 3-minute intervals until PaO_2 was below 80 mmHg and $PaCO_2$ above 40 mmHg at the following ventilator settings: PIP/PEEP 25/5 cmH_2O, rate 40 breaths/min, I/E ratio 1:2 and FiO_2 1.0

Experimental protocol

Within 10 minutes after the last lavage the animals were randomly allocated to one of the following treatment groups (n=8 each) and ventilated for 5 hours. FiO_2 was kept at 1.0 during all experiments.

PPV_{OLC} group.

In this group PEEP was increased to 15 cmH_2O, the ventilatory rate was increased to 120 breaths/min and the I/E ratio was set at 1:1. A pressure amplitude (PIP minus PEEP) was initially set at 10-12 cmH_2O in order to prevent hypercapnia. While continuing PPV, collapsed alveoli were recruited by increasing PIP in steps of 5 cmH_2O for a short period of time (10 s). This recruitment maneuver using incremental PIP was continued until an arterial oxygen tension/fractional inspired oxygen (PaO_2/FiO_2) $\geq$ 450 was reached, signifying optimal alveolar recruitment ("the open lung"). After the recruitment maneuver, PEEP was decreased in steps of 1 cmH_2O every 2-3 minutes until the PaO_2/FiO_2 ratio dropped below 450, indicating increased intrapulmonary shunt due to progressive alveolar collapse.

After identifying this closing pressure, collapsed alveoli were once again recruited and PEEP was set 2 cmH_2O above the closing pressure. The pressure amplitude was set to keep the $PaCO_2$ within the target range (30–45 mmHg).

$HFOV_{OLC}$ group.

This second OLC group was ventilated with HFOV (model 3100; SensorMedics Critical Care, Yorba Linda, CA, USA) using a frequency of 10 Hz and an I/E ratio of 1:2. The lung was opened by increasing the continuous distending pressure (CDP) during oscillation in steps of 5 cmH_2O for a short period of time (10 s) until a $PaO_2/FiO_2 \geq 450$ was reached. After this recruitment procedure, the CDP was lowered in steps of 1 cmH_2O every 2-3 min until alveolar collapsed resulted in a PaO_2/FiO_2 ratio below 450 (closing pressure). The lung was

once again opened and the CDP was set 2 cmH_2O above the closing pressure. The oscillatory pressure amplitude was set at a level which kept the $PaCO_2$ within the target range.

PPV_{CON} group.

This third group was ventilated in the pressure controlled mode applying a conventional strategy. As preliminary experiments showed that it was not possible to achieve a target PaO_2 ≥ 450 mmHg, the ventilator settings after the lavage procedure were adjusted to prevent critical hypoxia (PaO_2 < 60 mmHg). First, the I/E ratio was set at 1:1 and, if necessary, PIP was increased with a limit of 35 cmH_2O. If the level of PIP exceeded 30 cmH_2O, PEEP was increased to 6 cmH_2O.

The rate was preferentially changed (between 25–60 breaths/min) in order to keep the $PaCO_2$ within the target range. If adjusting the rate proved unsuccessful in controlling $PaCO_2$, PIP could be altered as long as the changes did not compromise PaO_2.

Ventilator settings and pressures, mean arterial blood pressure, heart rate and acid base status were recorded at the end of the instrumentation period, at the end of the lavage procedure and hourly after randomization. Although blood gas values were recorded continuously reference samples were drawn at these same time points (ABL 505, Radiometer, Copenhagen, Denmark).

Broncho-alveolar lavage procedure

After the 5 h ventilation period animals were killed with an overdose of pentobarbital. BAL was performed with saline-$CaCl_2$ 1.5 mmol/L (40 ml/kg). The amount of fluid collected was measured in order to calculate the percentage of lung lavage fluid recovered.

The recovered volume of the lavage procedure was filtered through a 100 μm sterile gauze to remove mucus. The BAL sample was centrifuged at 1400 rpm for 5 minutes at 4°C. The fluid fraction was separated from the cellular fraction and stored in aliquots at -80°C until analysis.

Total cells and PMN counts in BAL fluid

The number of cells obtained with the BAL procedure was determined using a hemocytometer. From the cellular fraction cytospin preparations were made and stained with Diff-Quick® (Dade Behring AG, Düdingenn, Switzerland) and cellular differentials were determined by counting 300 cells per cytospin.

Analysis of IL-8 and TNF-α in BAL fluid

To measure TNF- α and IL-8 concentration, BAL fluid was centrifuged (15000 rpm; 4°C; 20 min) to remove debris. Hereafter, BAL fluid was concentrated 20 times using centrifugal

ultrafilters with a molecular weight cut-off of 3kD (Centrex® UF; Schleicher & Schuell, Dassel, Germany).

TNF-α and IL-8 levels were determined in duplo by ELISA (TNF-α and IL-8 Swine Cytoscreen™ immunoassay kit; Biosource International, Camarillo, CA) according to the manufacturer. Cytokine levels in BAL fluid were expressed as pg/ml BAL fluid.

Analysis of thrombin activity in BAL fluid

Thrombin activity was determined using the thrombin specific chromogenic substrate Tos-Gly-Pro-Arg-pNa (Sigma, St Louis, MO) as described by Abildgaard and Lottenberg [21, 22]. Briefly, 25 µL of BAL fluid was diluted in 25 µL Tris-buffered saline (TBS; pH 8.3) and 25 µL BAL fluid was diluted in TBS containing 4.10^{-6} M of the thrombin inhibitor PPACK. These mixtures were added to a 96 well microtitre plate and incubated for 20 minutes at 37 °C to allow thrombin-PPACK complexes to form. Thereafter, 50 µL of 1 mM Tos-Gly-Pro-Arg-pNa (in 1.5 mM HCL) was added to the BAL fluid dilutions and incubated at 37 °C. The optical density (OD) was measured at 405 nm for a period of 6 hours. During this time period a linear increase in OD of the BAL sample was found (data not shown). Thrombin activity in every BAL sample is expressed as an OD value at 405 nm, which was determined as the difference in OD, measured at 6 h, between the BAL sample without and with PPACK. As a control for the assay 1.10^{-6} M of thrombin (Sigma, St Louis, MO) was also added to the substrate. This resulted in an OD, which was never reached by any of the BAL fluid samples. Preincubation of thrombin with PPACK resulted in complete inhibition of thrombin activity.

Statistical Analysis

Statistical analysis was performed using SPSS version 10 (SPSS Chicago, IL, USA). Data showing a normal distribution were analyzed with the Student's t test or the analysis of variance (ANOVA). If ANOVA resulted in $p < 0.05$ a Tukey post-hoc test was performed. Skewed data were analyzed using the Kruskal-Wallis test. A $p < 0.05$ was considered statistically significant.

RESULTS

All animals survived the study period. There were no intergroup differences in age, weight and number of lavages needed to induce lung injury.

Hemodynamic parameters as presented in table 1 showed no differences in mean arterial blood pressure and heart rate between the three ventilation groups. Due to differences in $PaCO_2$, the PH was generally higher in the PPV_{CON} group but there were no differences in bicarbonate levels.

Table 1. Data on circulatory parameters (mean ± SD) in the different study groups.

		H	L	1 h	2 h	3 h	4 h	5 h
MAP, mmHg	PPV_{OLC}	72±12	89±12	75±11	75±11	69±12	68±11	68±14
	PPV_{CON}	65±14	67±13	76±20	69±18	63±15	59±14	60±15
	$HFOV_{OLC}$	70±12	74±16	76±10	71±13	69±13	64±12	63±10
HR, b/min	PPV_{OLC}	145±14	174±29	195±32	193±29	190±38	194±36	200±40
	PPV_{CON}	137±17	157±20	191±29	198±33	198±36	206±38	212±33
	$HFOV_{OLC}$	151±18	190±32	179±30	195±37	206±35	210±42	203±35
PH	PPV_{OLC}	7.50±0.07	7.29±0.04	7.43±0.06	7.41±0.05	7.40±0.05	7.39±0.06	7.40±0.04
	PPV_{CON}	7.53±0.07	7.33±0.07	7.51±0.04[a]	7.48±0.07	7.47±0.07	7.45±0.09	7.45±0.08
	$HFOV_{OLC}$	7.50±0.07	7.27±0.07	7.44±0.06	7.42±0.06	7.43±0.06	7.41±0.07	7.41±0.05
HCO_3, mmol/L	PPV_{OLC}	27.6±2.6	21.5±2.6	23.3±2.1	23.1±2.5	23.1±2.2	23.3±2.7	23.3±2.1
	PPV_{CON}	27.5±2.9	23.3±2.6	21.3±1.8	20.5±1.4	20.5±1.1	21.3±2.8	22.9±2.2
	$HFOV_{OLC}$	25.±2.7	19.0±3.8	21.1±1.6	21.8±2.7	21.6±2.5	21.1±3.3	21.6±3.0

[a] $p < 0.05$ vs PPV_{OLC} and $HFOV_{OLC}$ group. ***MAP***, mean arterial bloodpressure. ***HR***, heart rate. ***H***, healthy. ***L***, lavaged

Table 2 summarizes the data on ventilator settings and gas exchange during the experiment. It shows comparable PaO_2 values in all three ventilation groups during the healthy pre-lavage period and also the post-lavage period. However, after randomization PaO_2 was significantly higher at all time points in both OLC groups compared with the PPV_{CON} group. Although $PaCO_2$ tended to be somewhat lower during the first few hours in the PPV_{CON} group, there were no differences compared with the other groups during the remainder of the experiments. The mean airway pressure (MawP) was comparable in all three ventilation groups, with clear differences between PIP and PEEP in both PPV groups.

Table 2. Data on ventilatory parameters and gas exchange (mean ± SD) in the different study groups.

		H	L	1 h	2 h	3 h	4 h	5 h
MawP, cmH_2O	PPV_{OLC}	4.5±0.5	10.3±1.0	14.6±3.3	14.6±3.0	14.2±2.9	14.1±2.9	13.7±2.9
	PPV_{CON}	4.3±0.5	10.1±0.6	14.3±1.8	14.1±1.9	14.1±1.8	14.3±1.8	14.3±1.8
	$HFOV_{OLC}$	4.1±0.4	10.4±1.1	13.8±2.6	13.7±2.4	13.5±2.7	13.3±2.9	13.2±2.8
PIP, cmH_2O	PPV_{OLC}[a]	10.4±0.9	25.0±0.0	18.8±4.7	18.8±4.4	18.6±3.9	18.5±3.9	17.9±3.5
	PPV_{CON}	9.9±0.6	25.0±0.0	26.6±1.5	26.5±1.5	26.5±1.5	26.8±1.6	26.8±1.6
	$HFOV_{OLC}$	10.3±1.7	25.0±0.0	-	-	-	-	-
PEEP, cmH_2O	PPV_{OLC}[a]	2.6±1.2	5.0±0.0	10.9±2.4	10.8±2.4	10.4±2.3	10.2±2.3	9.8±2.3
	PPV_{CON}	2.3±0.7	5.0±0.0	5.4±0.6	5.3±0.5	5.2±0.4	5.3±0.5	5.3±0.5
	$HFOV_{OLC}$	2.2±0.7	5.0±0.0	-	-	-	-	-
PaO_2, mmHg	PPV_{OLC}[a]	463±44	47±8	464±40	461±43	473±50	478±50	499±55
	PPV_{CON}	461±47	37±8	77±66	85±60	85±60	75±60	104±89
	$HFOV_{OLC}$[a]	458±74	40±14	470±74	491±39	517±33	519±55	517±53
$PaCO_2$, mmHg	PPV_{OLC}	36±4	47±3	36±5[b]	37±3[b]	38±3[b]	39±4	38±4
	PPV_{CON}	34±6	45±6	26±5	28±6	28±6	31±9	33±8
	$HFOV_{OLC}$	33±4	42±5	33±4[b]	32±6	32±6	34±5	35±5

[a] $p < 0.001$ vs PPV_{CON} group during the 5 h ventilation period. [b] $p < 0.05$ vs PPV_{CON} group. ***MawP***, mean airway pressure. ***PIP***, peak inspiratory pressure. ***PEEP***, positive end-expiratory pressure. ***H***, healthy. ***L***, lavaged

The percentage of BAL fluid recovered was 66 ± 6, 67 ± 5 and 68 ± 6 in the PPV_{OLC}, $HFOV_{OLC}$ and PPV_{CON} group respectively; the differences were not significant.

The cells detected in the BAL fluid after 5 h ventilation were predominantly alveolar macrophages and PMN. The numbers of both total cells and PMN were significantly higher in the PPV_{CON} group compared with both OLC groups (figure 1).

The values of IL-8 were significantly lower in the PPV_{OLC} group compared to the PPV_{CON} group (figure 2).

Although not significantly different there was also a trend to reduced levels of IL-8 in the $HFOV_{OLC}$ group compared to the PPV_{CON} group. No differences in IL-8 were observed between both OLC groups.

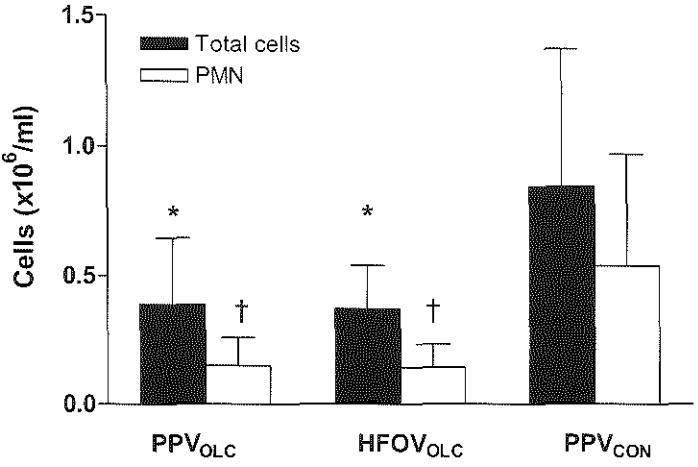

Figure 1.
Total cell count and the number of PMN (mean ± SD) in the three ventilation groups. * $p < 0.05$ and † $p < 0.02$ vs PPV_{CON} group.

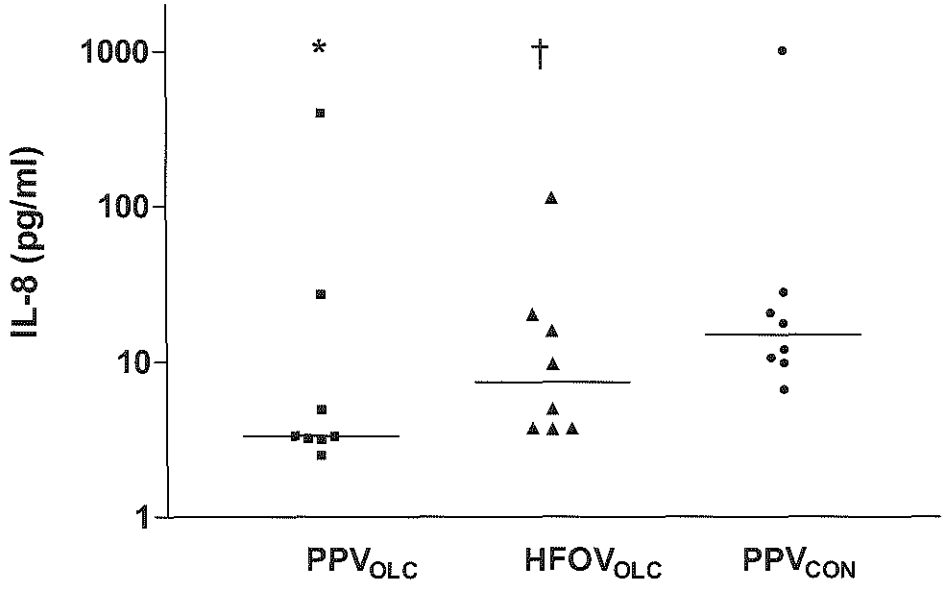

Figure 2.
Scatter plot of individual IL-8 values in the three ventilation groups. Median is presented as a solid line.
* $p < 0.05$ vs PPV_{CON} group, † $p = 0.1$ vs PPV_{CON} group

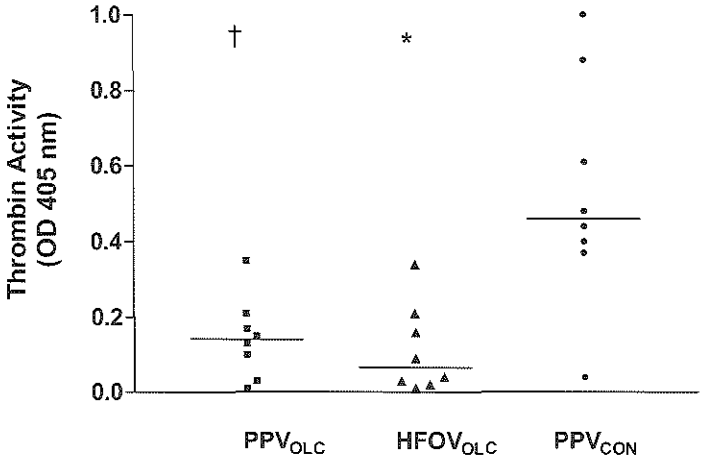

Figure 3.
Scatter plot of individual thrombin activity in the three ventilation groups. Median is presented as a solid line.
* $p < 0.05$ and † $p = 0.06$ vs PPV_{CON} group

Thrombin activity was significantly lower in the $HFOV_{OLC}$ group compared to the PPV_{CON} group (figure 3). There were generally also lower values in the PPV_{OLC} group, although the difference only approached significance ($p = 0.06$). Thrombin activity was comparable in both OLC groups.

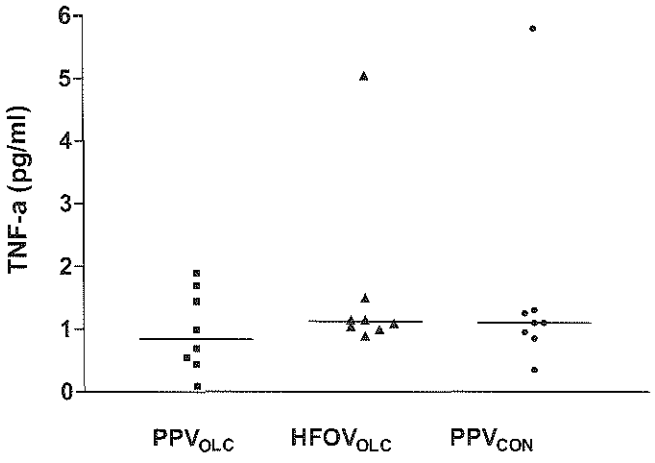

Figure 4.
Scatter plot of individual TNF-α values in the three ventilation groups. Median is presented as a solid line.

Although TNF-α was detected in most animals, levels showed no significant differences between the three ventilation groups (figure 4).

DISCUSSION

The present study shows that applying the OLC during PPV in surfactant-depleted newborn piglets reduces the alveolar influx of PMN, the concentration of IL-8 and the thrombin activity, compared with conventional PPV. It further shows that this attenuation of pulmonary inflammation is comparable to HFOV with OLC.

Only two recent studies in adult animals showed that the OLC can also be successfully applied during PPV [18, 19]. PPV_{OLC} resulted in optimized gas exchange and a reduction of ventilator-induced lung injury expressed as alveolar protein leakage, lung mechanics and histological damage. However, neither of these studies explored inflammatory changes in the lung and neither included a control group ventilated with conventional PPV.

Our present study confirms the superior gas exchange during PPV_{OLC} compared to PPV_{CON}, even though MawP was comparable in both groups. More important the present study also shows a reduction in pulmonary inflammation during PPV_{OLC}. First of all, PMN were significantly reduced in the alveolar wash during PPV_{OLC} compared to PPV_{CON}. This was also the case for the $HFOV_{OLC}$ group making this finding consistent with previous animal studies comparing the OLC during HFOV to conventional PPV [16, 17].

It has been proposed that activated PMN can cause tissue damage through the release of proteases, the production of reactive oxygen species and the release of cytokines [23]. This critical role of PMN in the pathogenesis of ventilator-induced lung injury is further emphasized by the experiments of Kawano et al., reporting relatively well preserved gas exchange, minimal protein leakage and absence of hyaline membrane formation during injurious conventional PPV of granulocyte depleted rabbits; this in contrast to rabbits with a normal granulocyte state [24].

These latter findings suggest that PMN not only migrate into the lung during injurious ventilation but also participate in the actual damage of lung tissue. Human data seem to support these animal findings, as several studies in newborn infants developing CLD showed a higher and persistent influx of PMN in BAL aspirates [4, 5].Besides a reduction in PMN, the present study also showed significantly reduced levels of IL-8 in the BAL fluid of animals ventilated with PPV_{OLC}. Although not statistically significant there was also a trend

to lower values of IL-8 in the $HFOV_{OLC}$ group. As a pro-inflammatory cytokine, IL-8 is considered one of the most potent chemotactic factors for PMN in the lung and is also capable of activating PMN by promoting neutrophil degranulation [25, 26]. Analyses of BAL fluid in newborn infants suffering from RDS have also shown IL-8 to play an important role in the progression to CLD [6, 7].

Thrombin activity was significantly lower in the $HFOV_{OLC}$ group compared to the PPC_{CON} group. Although not statistically significant, this reduction was also observed in the PPV_{OLC} group. Thrombin is known as an important enzyme in the coagulation cascade where it acts upon soluble fibrinogen to form insoluble fibrin [27]. Because the hyaline membranes, as described in histological reports of RDS, are in a large part composed of fibrin, the role of thrombin in acute lung injury has been further explored. It has been shown that thrombin increases vascular permeability and the production of cytokines like IL-8, thus further enhancing the inflammatory process in acute lung injury [28, 29]. Activation of the coagulation system and increased procoagulant activity of BAL fluids has been documented in newborns with RDS [30, 31]. These findings indirectly indicate a possible role of thrombin in RDS, although thrombin activity in BAL fluids was not measured in these studies.

Another important proinflammatory cytokine is TNF-α, which is known to upregulate the inflammatory process. It increases expression of IL-8 and is capable of degranulating neutrophils [26, 31, 32]. In the present study there was no significant difference in TNF-α between the three treatment groups. Up to now, reports on the role of TNF-α in the process of pulmonary inflammation and ventilator-induced lung injury have been contradictory. Several studies reported increased levels of TNF-α in the alveolar wash after injurious ventilation [17, 33], while others could not confirm these findings [34-36]. It is important to realize that comparison of these studies is difficult because most of them differ in animal model (ex vivo or in vivo), lung condition (healthy or preinjured), type of lung injury (LPS or wash-out), the expression of TNF-α (RNA or protein), the amount of mechanical induced injury to the lung and the duration of this insult. Based on these conflicting results some have suggested that injurious ventilation will only lead to activation of TNF-α if the lung is "primed" or preinjured before the mechanical insult [35]. Data on human newborn infants also showed increased levels of TNF-α in the alveolar compartment and reported an association with the development of CLD [9, 10]. However, these samples were taken after several days of

ventilation in contrast to several hours of ventilation in most animal studies. More studies are needed to further elaborate the role of TNF-α in ventilator-induced lung injury.

Another important finding in the present study is the comparable effect of both PPV_{OLC} and $HFOV_{OLC}$ on reducing pulmonary inflammation. This finding once again shows that it is not the ventilation mode (PPV or HFOV) but the ventilation strategy (OLC) that is important in the attenuation of ventilator-induced lung injury, as suggested by others [37].

Although others have shown that lung recruitment with a few large breaths can augment lung injury, our study shows no increased injury in terms of inflammation after alveolar recruitment in the PPV_{OLC} group [38]. Although this discrepancy could be explained by the animal model used, another possible explanation for these different findings could be the fact that we used high levels of PEEP during the recruitment phase. This way we prevented repetitive collapse and reexpansion of alveoli during recruitment, which has been shown to be harmful to the surfactant deficient lung [14]. Although HFOV has been widely implemented in the treatment of ARF in neonates, the majority is still ventilated with conventional PPV [39]. As in our PPV_{CON} group, PEEP seldom exceeds 5–6 cmH_2O and active recruitment maneuvers using high levels of PIP are not performed during conventional PPV of neonates admitted to the neonatal intensive care units. Our study shows that this can be potentially harmful to the surfactant-deficient newborn lung. Although HFOV is effective in reducing CLD in preterm infants there are concerns regarding the development of intracerebral hemorrhage [40]. Fortunately, two recent large randomized trials showed no increased incidence of intracranial hemorrhage during HF(O)V in preterm infants [41, 42]. Nevertheless when implementing PPV_{OLC} in preterm infants careful monitoring of possible cerebral side effects of using high levels of PEEP is imperative.

In conclusion, our study shows that application of the OLC during PPV optimizes gas exchange and attenuates pulmonary inflammation. These results further strengthen the current ideas that inflammation plays an important role in ventilator induced lung injury and sub-sequent respiratory morbidity such as CLD and that these aspects can be influenced by type of ventilation. Because there were no differences between PPV_{OLC} and $HFOV_{OLC}$, these results further emphasize that the ventilation strategy is more important than the ventilation mode.

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Chapter 9

PEEP influences *Klebsiella pneumonia* outgrowth and translocation in ARF/ARDS lungs

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Submitted

ABSTRACT

Acute respiratory failure (ARF) increases susceptibility for pneumonia we examined the influence of ventilation with the Open Lung Concept (OLC) on bacterial outgrowth in the lung and also translocation to the systemic circulation in *Klebsiella pneumoniae* infected surfactant deficient rats. ARDS was induced by surfactant depletion, followed by ventilation according to the OLC (peak inspiratory pressure (PIP)/PEEP; 35/18 cmH_2O) or with 'normal' PEEP (PIP/PEEP; 30/10 cmH_2O). A third healthy group, was ventilated with the 'normal' PEEP level of 10 cmH_2O, a fourth group of animals was not infected and was not-ventilated and served as controls. Outgrowth of *K. pneumoniae* from lungs and blood was determined for all infected animals, furthermore some components of pulmonary host defense system, such as neutrophil recruitment and cytokine production were assessed.

In both ALI/ARDS groups all animals had positive blood cultures after 3 hours of ventilation. However, in the ARDS group ventilated with the OLC outgrowth was significantly reduced to a level similar to the infected ventilated healthy group. TNF-α and MIP-2 levels were higher in the lungs in the normal PEEP group correlating with increased bacterial outgrowth. These experimental data show that the ventilation strategy influences the pulmonary and general infection level of animals as well as the inflammatory process in the lung. One may speculate that in septicemia due to pneumoniae outcome is related to PEEP levels.

INTRODUCTION

The applied ventilation strategy directly influences outcome, especially of patients suffering from acute respiratory distress syndrome (ARDS) [1-3]. For example so-called 'lung protective' ventilation strategies, using lower tidal volumes (V_T) combined with higher levels of positive end-expiratory pressure (PEEP) have demonstrated to reduce mortality in ARDS patients [1-3].

Interestingly, these strategies decreased circulating levels of pro-inflammatory cytokines [2, 3] and subsequently reduced the incidence of multiple organ failure (MOF) [4] the major cause of mortality in ARDS patients [5]. It is thought that these decreased levels of circulating cytokines are caused by a decline in local production. It has been demonstrated that mechanical ventilation alters the pulmonary host defense and thereby increases the susceptibility for an infection [6]. Available data, both experimental and clinical, suggest that ventilated ALI/ARDS lungs are particularly susceptible to develop pneumonia [7] and that patients with pneumonia often develop septicemia which together with MOF is the main cause of death in ARDS patients.

Previously it has been reported that when a lung with an existing pneumonia is ventilated application of 10 cmH_2O of PEEP reduced bacterial translocation from the lung to the blood. We hypothesised that mechanical ventilation with a strategy of active lung recruitment and stabilization with high PEEP (18 cm H_2O), the Open Lung Concept (OLC) enhances clearance of bacteria and decreases spread of pneumonia in ARDS lungs [8]. Therefore, outgrowth of *K. pneumoniae* from lungs and blood was determined in an ARDS model, during ventilation with the OLC [8] and during normal PEEP. Furthermore, we investigated some elements of pulmonary host defense, such as neutrophil recruitment (MPO) and cytokine production.

MATERIALS AND METHODS

Animals

The institutional Animal Investigation Committee Care approved the study protocol and handling of the animals was in accordance with the European Community guidelines.

A total of 48 male Sprague Dawley rats (body weight 270-320 g) was used. In two groups of

ventilated rats ARDS was induced followed by infection with *K. Pneumoniae* and by ventilation with either the OLC or conventional level with 'normal PEEP'. The third group of ventilated rats was only infected with *K. Pneumoniae* and ventilated with the normal level of PEEP. A fourth healthy group was not ventilated and not infected and served as controls. An open lung was characterized by a PaO_2 > 450 mmHg at 100% oxygen corresponding with a shunt of less then 10%

Surgical procedure

Anesthesia was induced with 65% nitrous oxide/33% oxygen/2% isoflurane (Isoflurane; Pharmachemie BV, Haarlem, The Netherlands), a sterile polyethylene catheter was inserted into a carotid artery for arterial blood sampling and a sterile metal cannula was inserted into the trachea. Anesthesia was replaced with pentobarbital sodium 60 mg/kg/h bodyweight i.p. (Nembutal®; Algin BV., Maassluis, The Netherlands). Subsequently muscle relaxation was induced and sustained with pancuronium bromide 2 mg/kg/h bodyweight i.m. (Pavulon®; Organon Technika, Boxtel, The Netherlands) followed by connection to the ventilator. Animals were ventilated in parallel in a pressure-controlled time-cycled mode (Siemens Servo 300, Siemens Elema, Solna, Sweden). The initial pressures were a peak inspiratory pressure (PIP) of 12 cm H_2O and a PEEP of 2 cm H_2O, the fractional inspired oxygen concentration (FiO_2) was set at 1.0, I/E ratio of 1:2; frequency = 30 breaths per minute. To re-aerate atelectatic lung areas induced by the surgical procedure, the airway pressure was increased to a PIP of 25 cm H_2O for 3 breaths. The body temperature was kept at 37°C by means of a heating pad.

Induction of ARDS

Twenty-four rats were surfactant depleted by whole lung lavage (33 ml warm saline/kg bodyweight) according to Lachmann [9] until PaO_2 was < 80 mmHg. During the lavage the ventilation pressures were set at a PIP of 26 cm H_2O and a PEEP of 6 cm H_2O. After the lavage procedure ventilator settings were set as follows: conventional ventilation; PIP/PEEP was increased to 30/10 and frequency to 50 bpm (n = 12; ARDS, normal PEEP; group 30/10 L), or OLC; PIP was increased to 45 cmH_2O to recruit atelectatic lung tissue for 5 breaths and subsequently PIP/PEEP was set to 35/18, frequency to 70 bpm and I/E ratio to 1:1 (n = 12; ARDS, OLC; group 35/18 L) [8]. A third experimental group was not lung lavaged and was ventilated with PIP/PEEP of 30/10, frequency 25 bpm (n = 12; non ARDS; group 30/10 H). Finally a fourth group of rats was neither ventilated, nor lavaged, nor inoculated with bacteria

(n = 12; controls; group healthy). Ventilation was adjusted in the ventilated groups to maintain normocapnia.

Induction of pneumonia

After adjusting ventilation settings according to one of the three experimental groups, the inoculum of *K. Pneumoniae* was aerosolised intratracheally using a miniature nebulizer (Penn-Century, Philadelphia, PA, USA). Animals were subsequently ventilated for 180 minutes.

The inoculum of 250 µl of saline with a concentration of 10^8 colony forming units (CFU)/ml *K. pneumoniae* was prepared as follows: stationary-phase cultures were prepared by incubation for 16 hours at 37°C in Mueller-Hinton broth (MHB) with a standard *K. pneumoniae* solution [10]. Thereafter, 200 µl of this broth was taken and combined with 19.8 ml MHB and incubated for 2 hours at 37°C followed by washing and dilution until the final concentration of 10^8 CFU/ml. The inoculum was stored on ice until use.

To verify the number of viable bacteria in the inoculum, 100 µl of 10-fold dilution steps in saline were plated on tryptone soya agar (TSA) plates. The TSA plates were incubated overnight at 37°C and CFUs were visually counted the following day.

Measurements

Arterial blood gases were measured with conventional methods (GEM, Premier 3000, Instrumentation Laboratory, Breda, The Netherlands) just before start of whole lung lavage (baseline value), immediately after lavage (lavage) and at 5, 60, 120 and 180 minutes after lavage. To replace the blood loss caused by sampling, animals received 4 ml/kg Hemohes 6% (B. Braun Melsungen AG, Melsungen, Germany) every hour.

Blood samples of 1 ml were taken before lavage and at 30 and 180 minutes after lavage, and cultured undiluted onto blood agar plates (Becton Dickinson bv., Alphen a/d Rijn, The Netherlands) and incubated at 37°C, *K. pneumoniae* CFU were counted after 24 hours, the lower limit of detection by this method was 1 CFU/ml.

After 180 minutes of ventilation animals of all three experimental groups were killed with an overdose of pentobarbital sodium. Lungs were taken sterile from the thorax, weighed and homogenised in 20 ml sterile saline at 4°C for 1 minute at 40,000 rpm in a tissue homogenizer (Virtis "23", The Virtis Company inc., N.Y., USA). The number of viable bacteria in the lung homogenates of the three experimental groups was determined by plating 10-fold dilution steps on TSA plates. These plates were incubated at 37°C, *K. pneumoniae*

CFU were counted after 24 hours, the lower limit of detection by this method was 250 CFU/lung.

Lung homogenates were centrifuged at 4°C at 400 *g* for 10 minutes to spin down cells and cellular debris, the supernatant was collected and stored at –80°C until further analysis. Heperanized blood samples were taken before each animal was killed. Supernatant of blood was centrifuged at 4°C at 400 *g* for 10 minutes to remove cells and cellular debris, snap-frozen on liquid nitrogen and stored at -80°C until further analysis.

MPO measurements

To determine the recruitment of neutrophils myeloperoxidase (MPO) activity in the supernatant was determined using a photospectrometer (Beckman DU 7400, Fullerton, CA, USA) at 450 nm with human MPO (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) as a standard as described by Wollin et al. [11].

Cytokine measurements

Systemic and local levels of tumor necrosis factor (TNF)-α, interleukin (IL)-10 and macrophage inflammatory protein (MIP)-2 were determined by rat specific enzyme-linked immunosorbent assay (ELISA) in accordance with the instructions of the manufacturer (R&D, Minneapolis, MN, USA).

Statistic analysis

Data on PaO_2, MPO and lung weight are reported as mean ± standard deviation (SD). Data for cytokine values are reported as mean ± standard error of mean (SEM). Log transformed CFU counts (lung and blood) and local and systemic cytokine levels were compared by means of ANOVA, with a Bonferroni post-hoc test. Comparisons for PaO_2, MPO and lung weight were performed by repeated measures ANOVA with a Bonferroni post-hoc test. Statistical significance was accepted at $p < 0.05$.

Table 1.
Data on myeloperoxidase (MPO) activity (lung homogenate) and lung weight, in the three experimental groups and in the non-ventilated controls (Healthy).

	30/10 L	35/18 L	30/10 H	Healthy
MPO (units/ml)	5.28 ± 2.3	6.79 ± 2.12	4.25 ± 2.04*	4.32 ± 1.11*
Lung weight (g/kg BW)	21.6 ± 2.3	21.4 ± 1.5	14.2 ± 1.5*#	8.7 ± 0.6*# †

Significant intergroup differences: * p<0.05 vs 35/18 L; # p<0.05 vs 30/10 L and † p<0.001 vs 30/10 H.

RESULTS

Induction of pneumonia

Macroscopic examination of the lungs showed severe atelectasis and edema formation in both ARDS groups; there were no hemorrhagic foci in any of the infected groups. The non-lavaged infected group showed no apparent lung damage. In group 30/10 L three animals died during the experiment due to shock-induced septicemia, values of CFU counts and cytokine measurements in the lungs and blood of these animals were excluded from analysis because it was impossible to obtain enough blood and due to uncertainty of the time between death and lung harvest. Lung weights of the ARDS animals (30/10 L, 35/18 L) were significantly higher than those of the non-lavaged infected but ventilated animals (30/10 H) (Table 1). Furthermore, lung weights of the non-ventilated healthy controls were significantly lower compared to the three experimental (ventilated and infected) groups.

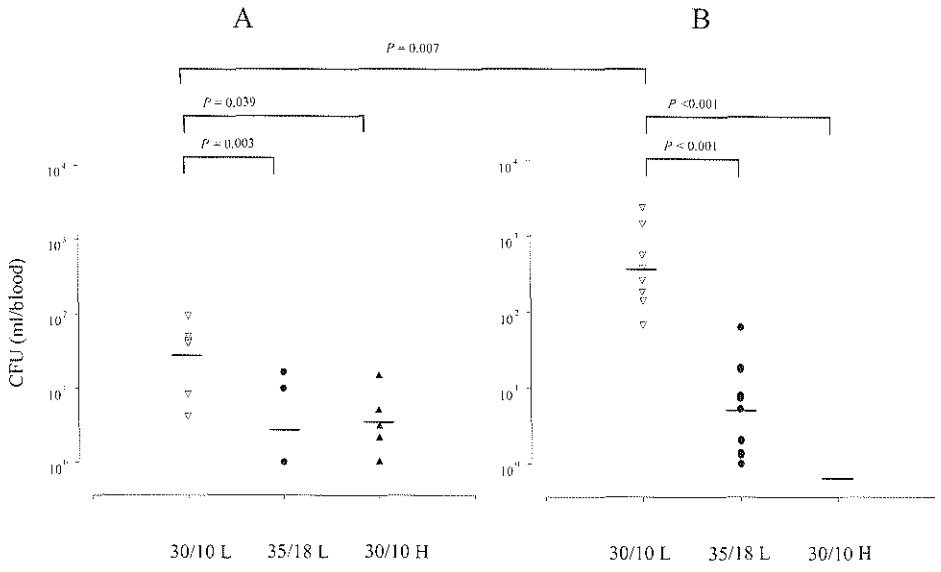

Figure 1 A and B.
CFU in blood samples taken 30 minutes (A) and 180 minutes (B) after inoculation with *K. pneumoniae*. Rats were lavaged and subsequently ventilated with a normal PEEP level (open triangle; group 30/10 L), or with a high PEEP level (closed circle; group 35/18 L). One group was mechanically ventilated and inoculated with bacteria without any lavage (closed triangle; group 30/10 H). Horizontal bars indicate mean value. *p*-values indicate significant differences between the three experimental groups.

Outgrowth of K. pneumoniae in lungs and blood

Thirty minutes after inoculation, in group 30/10 L 7 of the 12 (7/12) rats had positive blood cultures, whereas in groups 35/18 L and 30/10 H 5/12 and 5/12 rats, respectively, were bacteremic (Fig 1a). Thirty minutes after inoculation, the amount of CFU *K. pneumoniae* in blood was significantly higher in group 30/10 L than in the other two infected groups (p = 0.003 vs. 35/18 L, and p = 0.039 vs. 30/10 H, respectively). Interestingly the number of CFU in blood of group 35/18 L did not change between the two time points 30 and 180 minutes ventilation (p = 0.057), while none of the rats in group 30/10 H were bacteremic at 180 minutes. In contrast, the CFU counts in blood of group 30/10 L increased dramatically over time (p = 0.007; 30 min vs. 180 min) (Fig. 1b).

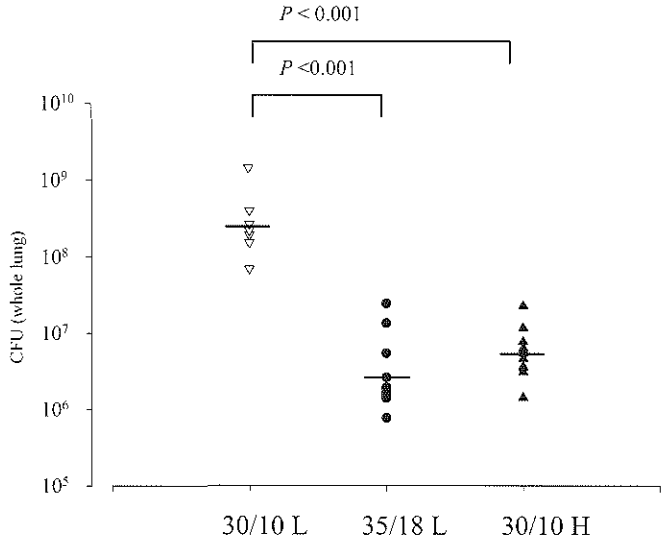

Figure 2.
Outgrowth of *K. pneumoniae* in lung homogenates. Rats were lavaged and subsequently ventilated with a normal PEEP level (open triangle; group 30/10 L; n=9), or a high PEEP level (closed circle; group 35/18 L; n=12). One group was mechanically ventilated and inoculated with bacteria without any lavage (closed triangle; group 30/10 H; n=12). Horizontal bars indicate mean value. p-values indicate significant differences between the three experimental groups.

Three hours after inoculation, group 30/10 L had significantly more *K pneumoniae* CFU in their lungs compared with groups 35/18 L and 30/10 H ($p < 0.001$ vs. 35/18 L, and $p < 0.001$ vs. 30/10 H) (Fig. 2). In the two latter groups CFU counts were similar.

Arterial oxygenation

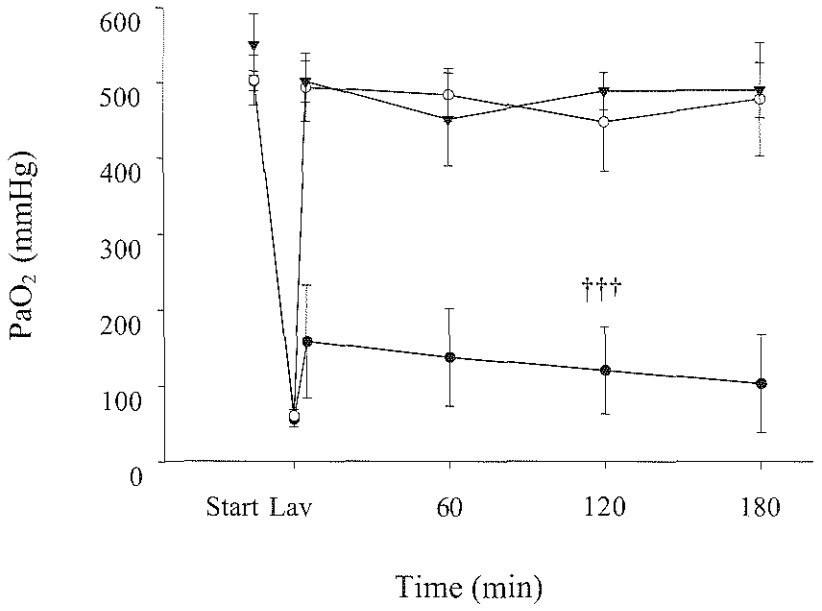

Figure 3.
Arterial oxygenation (PaO_2) in the three experimental groups over time. Start: baseline values. Lav: after lavage. Closed circle: group 30/10 L, open circle: group 35/18 L and closed triangle: group 30/10 H. ††† indicates death of 3 rats in the 30/10 L group. Significant intergroup differences are given in the text.

Figure 3 shows that arterial oxygenation (PaO_2) in groups 30/10 L and 35/18 L decreased after lavage; compared with 35/18 L and 30/10 H PaO_2 in group 30/10 L remained significantly lower at all time points during the study period ($p < 0.05$). After lavage and after adjusting the ventilator settings, PaO_2 in group 35/18 L increased to baseline values and remained at baseline values for the remainder of the experiment. In group 30/10 H oxygenation remained at baseline values during the whole experiment (Fig. 3).

Local MPO levels

MPO activity in the lungs was significantly higher in group 35/18 L compared to both the non-surfactant depleted group (30/10 H) and to the healthy controls (healthy) (Table 1).

Cytokines

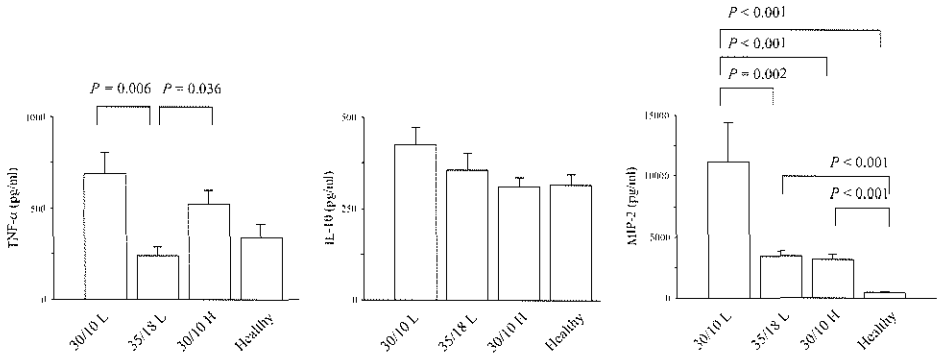

Figure 4.
TNF-α, IL-10 and MIP-2 production in lung homogenate in the three experimental groups and in the non-ventilated controls (Healthy). Significant intergroup differences are indicated in the figure.

Because local production of cytokines and chemokines within the pulmonary compartment can influence anti-bacterial host defense mechanisms during pneumonia [12-15], we measured the concentrations of TNF, IL-10, and MIP-2 in lung homogenates and serum after inoculation with *K. pneumoniae*. While there were no differences in TNF-α levels in plasma in the four study groups, local TNF-α levels were significantly higher in the lungs of rats in group 30/10 L and 30/10 H than in group 35/18 L (Figs. 4 and 5). Levels of IL-10 were similar in all four groups in both lung and plasma (Figs. 4 and 5). Levels of MIP-2 were significantly increased in the lungs of all ventilated animals compared to the non-ventilated control animals. Importantly, the levels of MIP-2 were significantly higher in group 30/10 L

compared to the other two groups of ventilated animals both in the lung and in the serum compartment, indicating spillover of MIP-2 (Figs. 4 and 5).

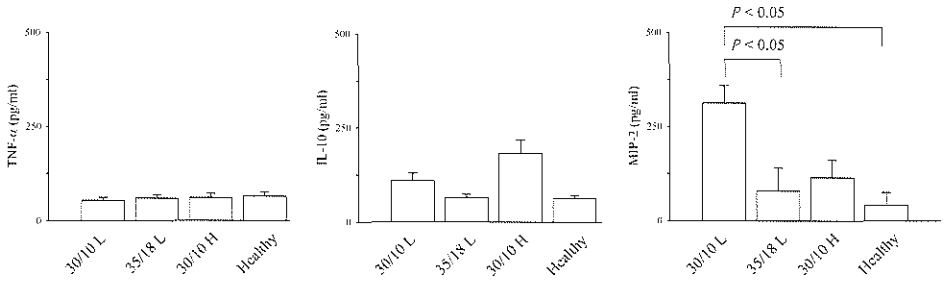

Figure 5.
TNF-α, IL-10 and MIP-2 production in serum in the three experimental groups and in the non-ventilated controls (Healthy). Significant intergroup differences are indicated in the figure.

DISCUSSION

In the present study we demonstrated that a ventilation strategy combining a recruitment procedure with a high PEEP level (OLC) increases bacterial clearance and reduces translocation of bacteria to the systemic circulation. Higher levels of TNF-α and MIP-2 in the lungs accompanied the increase in bacterial outgrowth, i.e., levels of these mediators were lower in the lungs ventilated with the OLC.

To our knowledge, the present study is the first study to investigate the role of a ventilation strategy on infection progression (*K. pneumoniae*) in an injured lung. The injured lung, especially in ARDS patients, is more susceptible for bacterial colonization [16]. We demonstrate that clearance of *K. pneumoniae* from ARDS lungs is influenced by the ventilation strategy applied. It is known that PEEP levels influence bacterial outgrowth in healthy lungs: Tilson and colleagues infected healthy dogs with *Pseudomonas aeruginosa*

and subsequently ventilated the animals for 24 hours without PEEP or with 10 cmH_2O of PEEP [17]. Compared with ventilation with 10 cmH_2O of PEEP, ventilation without PEEP resulted in a 100-fold increase in bacterial outgrowth from the lungs and a corresponding increase in the number of positive blood cultures. Importantly, a 75% mortality was demonstrated in the non-PEEP group versus no lethality in the group ventilated with 10 cmH_2O of PEEP [17]. Similar to the observations made by Tilson and colleagues, in our study the bacterial load in the lungs, the number of positive blood cultures, as well as mortality, were all higher in the group ventilated with a lower level of PEEP than in the group ventilated according to the OLC.

It is known that dissemination of bacteria to the blood is dependent on the overdistension applied to the lung [10, 18]. Nahum and colleagues ventilated healthy dogs with either 3 cmH_2O of PEEP (low PEEP, allowing repetitive alveolar collapse) or 10 cmH_2O of PEEP (high PEEP, preventing alveolar collapse) [18]. After instillation of *Eschericha coli* into the lungs in the low PEEP group significantly more positive blood cultures were found compared with the high PEEP group. In addition mortality was higher in dogs mechanically ventilated with 3 cmH_2O PEEP. Importantly, in this latter study the bacterial load was not quantified. Similarly, in a model of established pneumonia, Verbrugge *et al.* demonstrated that alveolar overdistension and repetitive alveolar collapse (due to absence of PEEP) resulted in a significant increase in positive blood cultures [10]. Although it seems feasible that the increase in bacteremia in the present study could have been caused by augmented translocation due to alveolar overdistension. In the present study, however a higher PIP of 35 cmH_2O in the high PEEP group was not associated with more translocation of bacteria to blood compared with a PIP of 30 cmH_2O in the normal PEEP group. This directly argues against the traditional hypothesis of bacteremia caused by overdistension alone due to high pressure [10, 18]. Therefore we belief that an additional explanation that due to decreased bacterial clearance inside the lung which increases the bacterial load in the lung, results in subsequent translocation of more *K. pneumoniae* to the systemic circulation.

When endogenous surfactant is still present, bacterial growth is hampered by the complete alveolar defense system, including surfactant and surfactant proteins, such as surfactant protein-A [19], as well as macrophages and neutrophils as observed in our non-surfactant depleted group (30/10 H). Application of a lung protective ventilation strategy reduces further damage to the lung and prevents further deterioration of the surfactant system [8].

This prevention of further damage to the already impaired endogenous surfactant system could further explain why there was less bacterial growth in our high PEEP group [18, 20]. The observation that there were no positive blood cultures in the 30/10 H group at 180 minutes reiterates the importance of surfactant as the rate-limiting factor in transfer of agents across the alveolar-capillary membrane [21, 22].

Similar to our observation, Broug-Holub and colleagues also observed in *K. pneumoniae* infected animals that a higher bacterial load was associated with increased mortality, high levels of TNF-α and MIP-2 and a corresponding increase of neutrophils; they also showed that the increased mortality was related to alveolar macrophage (dys)function [23]. Therefore, in our study another explanation for the decreased bacterial clearance in the normal PEEP animals could have been the reduction of macrophages by lung lavage. Applying a protective ventilation strategy with high PEEP levels might spare the residual macrophage function, because particularly bactericidal function is diminished in atelectatic lungs [20, 24, 25]. The alveolar milieu, especially in areas filled with edema (atelectasis) and low oxygenation, is a more suitable environment for bacterial growth due to decreased phagocytic activity of macrophages [20, 24, 25].

In conclusion, by optimizing mechanical ventilation in ARDS (reducing overstretching and repetitive collapse; e.g. shear forces) with high PEEP levels (the OLC), we demonstrated a reduced bacterial outgrowth and, more importantly, a reduction of bacterial translocation to the systemic circulation. Together with the results on cytokines, these data support the need for increasing awareness that the way we ventilate (ARDS) patients influences their outcome.

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SUMMARY AND CONCLUSIONS

Chapter 1 presents an overview on the effect of mechanical ventilation on the inflammatory response occurring in an ARDS lung. Ventilation itself is a major reason for progression of the inflamed state of the lung and the subsequent involvement of distal organs. Repetitive collapse and subsequent re-opening of alveoli by ventilation will generate 'shear forces'. Shear forces affect a much larger area of the lung than the alveolus subjected to the collapse, further propagating the damage. These shear forces will damage normal lung physiology leading to inactivation of the endogenous surfactant system, alterations in the function of the alveolar-capillary membrane and destruction of cells. Large tidal volumes and too low levels of positive end-expiratory pressure (PEEP) which are unable to stabilize alveoli are the predominant cause of these shear forces. When these shear forces are present a complex cascade of inflammation is activated both intra-cellularly and extra-cellularly.

The primary cause of death in ARDS is multi-organ failure and sepsis and not hypoxemia. Ranieri and colleagues showed that applying a lung protective ventilation strategy reduces the levels of pro-inflammatory cytokines in both the lung and the serum, correlating with less organ failure and lower mortality in these patients. In **Chapter 2** we demonstrate that injurious mechanical ventilation results in loss of a compartmentalized inflammatory response. The production of tumor necrosis factor-α (TNF-α), an early response pro-inflammatory cytokine, is compartmentalized. Mechanical ventilation induces a shift of high cytokine levels to the adjacent compartment (e.g. from the lung to the serum and vice versa). Preventing injurious ventilation by application of a sufficient level of PEEP reduced this loss of compartmentalization.

The loss of compartmentalization is dependent on the barrier function of the alveolo-capillary membrane. In **Chapter 3** we explored the essential role of surfactant on the homeostasis of the alveolo-capillary membrane. Increasing the intra-alveolar amount of surfactant to supra-physiological conditions followed by ventilation with the same injurious strategy used in Chapter 2 reduced the decompartmentalization of TNF-α. The presence of a sufficient amount of active surfactant helped stabilize transfer of cytokines across the alveolo-capillary membrane. Combining exogenous surfactant with a sufficient level of PEEP further enhanced the effect of surfactant as a rate limiting factor.

An ongoing controversy exists on the role of ventilation-induced mediator release in a 'healthy' non-inflamed lung. In **Chapter 4** we demonstrate that some mediators are released even in non-inflamed lungs by injurious mechanical ventilation; especially macrophage inflammatory protein (MIP)-2 and interleukin (IL)-6.

Before mediators are released by mechanical ventilation the stimulus (either by mechanical or biological stretch/pressure) must activate certain pathways to elicit a response in the lung cells. In **Chapter 5** we demonstrate that ventilation with high distending pressure activate mitogen activated protein kinases (MAPK) *in vivo*. Enhanced phosphorylation (e.g. activation) was observed within 60 min of ERK-1/2, JNK, c-Jun, Elk-1 and ATF-2 compared to unventilated or normally ventilated rats. Furthermore, the transcription factor NF–κB pathway was also activated by high pressure ventilation. Both MAPK and NF–κB pathways may contribute to pulmonary inflammation and proliferation.

In **Chapter 6** we prolonged the overdistension in a healthy lung by increasing the amount of surfactant to supra-physiological levels. During mechanical ventilation with high distending pressures active surfactant is squeezed out to the distal airways, resulting in a decrease of stretch of lung tissue when the same airway pressures are applied. In this study additional active surfactant was instilled into the lung to keep the stretch applied to the lung constant by compensating this squeeze-out phenomenon. This prolongation of distensibility while simultaneously keeping the peak inspiratory pressures constant led to similar activation of the MAPK pathway as observed in the study in Chapter 5. There was also an increase in systemic release of MIP-2 and IL-6 when lungs were subjected to high distending pressures similar to the observations made in the study in Chapter 4. Prolonging the distensibility in a normal lung, however, did not result in a significant further activation of the MAPK pathway or the release of MIP-2 and IL-6. Prolonging the distensibility with exogenous surfactant in a healthy lung, however, did lead to increased release of TNF-α.

In **Chapter 7** we observed that injurious mechanical ventilation led to increased cytokine mRNA expression in an inflamed lung. Heat shock proteins (HSP) are produced in cells when they are exposed to a variety of harmful stimuli (hypoxia, heat). In this study we show that injurious mechanical ventilation can also induce HSP production.

Amato and colleagues demonstrated that a lung protective ventilation strategy which combines an active recruitment procedure with a level of PEEP sufficient to stabilize recruited lung tissue and as low as possible tidal volumes, reduced mortality in ARDS

patients. In **Chapter 8** we studied the effect of a similar lung protective ventilation strategy (Open Lung Concept) on inflammation markers in surfactant-deficient piglets compared to conventionally ventilated animals. Application of the Open Lung Concept not only improved arterial oxygenation and lung mechanics but also reduced influx of polymorphonuclear neutrophils into the lung and reduced levels of interleukin-8. Furthermore, thrombin activity of the animals ventilated with the Open Lung Concept was also significantly lower.

Finally, in **Chapter 9** we show that lung protective ventilation reduces bacterial growth and dissemination of the bacteria to the blood. Infection with *Klebsiella pneumoniae* increased inflammation of the lung and increased accumulation of neutrophils into the lung. When surfactant was removed from the lung bacterial proliferation increased, the lack of surfactant altered the barrier function of the alveolo-capillary resulting in a progressive increase of positive blood cultures. Barrier function could, however, be compensated by using a lung protective ventilation strategy.

In conclusion, in this thesis we show the essential role mechanical ventilation has on propagation of the inflammation in an ARDS lung. Mechanical ventilation affects the inflammatory response at several levels, from the stretch-induced activation of intracellular messenger such as MAPK and NF–κB, to the activation of mRNA and subsequent release of several inflammatory cytokines. Furthermore, ventilation disrupts the normal barrier function of the alveolo-capillary membrane resulting in loss of compartmentalization of cytokines. Finally, even bacterial growth/colonization is directly influenced by mechanical ventilation. Thus when ventilation is necessary, especially in injured lungs (e.g. ARDS), the choice how to ventilate these lungs directly influences the whole cascade of inflammation and finally patient outcome.

SAMENVATTING EN CONCLUSIES

In **Hoofdstuk 1** wordt een overzicht gegevenvan; hoe in een ARDS long mechanische beademing de immuunreactie beïnvloedt. Beademing op zich is een belangrijke oorzaak dat de inflammatie van de long immuunactivatie geeft in distale organen. Het herhaaldelijk samenvallen en het vervolgens weer openen van alveoli tijdens beademing veroorzaakt zogenaamde 'shear forces'. De effecten van shear forces beslaan een veel groter oppervlakte dan alleen de alveolus die samenvalt, hetgeen resulteert in progressie van de schade. Shear forces beschadigen de normale longfysiologie wat leidt tot inactivatie van het endogene surfactant systeem, permeabiliteit-verandering van de alveolaire-capillaire membraan en cel destructie. Te hoge slagvolumes en een positief eind-expiratoire druk (PEEP) die te laag is om de alveolus te stabiliseren, zijn de belangrijkste oorzaken die deze shear forces genereren. Wanneer shear forces gegenereerd worden, activeren deze een complexe intra- en extracellulaire immunologische cascade.

De primaire doodsoorzaak van ARDS-patiënten is multi-orgaanfalen en sepsis, en niet hypoxie. Ranieri en collega's hebben aangetoond dat een longbeschermende beademingsstrategie verlaging geeft van de hoeveelheid pro-inflammatoire cytokines zowel in de long als ook in het serum. Deze verlaging correleert met een reductie van de incidentie van orgaanfalen en dit geeft een vermindering van mortaliteit in deze patiënten populatie.

In **Hoofdstuk 2** tonen we aan dat, schadelijke mechanische beademing verlies van een gecompartimentaliseerde immuunreactie veroorzaakt. Tumor necrosis factor-α (TNF-α), een vroege respons pro-inflammatoir cytokine, wordt gecompartimentaliseerd geproduceerd. Mechanische beademing induceert verschuiving van een hoge locale concentratie van dit cytokine naar het ernaast gelegen compartiment (in andere woorden van de long naar het serum en vice versa. Het voorkomen van beademingsschade door beademing met voldoende PEEP reduceert dit verlies van compartimentalisatie.

Het verlies van compartimentalisatie is afhankelijk van de barrièrefunctie van de alveolaire-capillaire membraan. In **Hoofdstuk 3** onderzoeken we de essentiële rol van surfactant op de alveolaire-capillaire homeostasis. Het verhogen van de intra-alveolaire hoeveelheid surfactant tot een supra-fysiologisch niveau, gevolgd door beademing met dezelfde schadelijke beademingsstrategie zoals beschreven in Hoofdstuk 2, vermindert de decompartimentalisatie van TNF-α. De aanwezigheid van voldoende actief surfactant verlaagt de transfer van

cytokines over de alveolaire-capillaire membraan. De combinatie van exogeen surfactant met voldoende PEEP versterkt het effect van surfactant als beperkende factor in de transfer over het alveolaire-capillaire membraan.

Er bestaat nog steeds controverse over de rol van beademing geïnduceerde mediator activatie in gezonde niet-geïnflammeerde longen. In **Hoofdstuk 4** tonen wij aan dat, tijdens mechanische beademing sommige mediatoren vrijkomen in de niet-immuun geactiveerde long, met name macrophage inflammatory protein (MIP)-2 en interleukine (IL)-6.

Voordat er mediatoren vrijkomen door mechanische beademing moet de stimulus (mechanisch of biologisch rek/druk) specifieke intracellulaire cascades activeren in de longcellen. In **Hoofdstuk 5** laten we zien dat beademing met drukken die overrekking veroorzaken, activatie geven van mitogen activated protein kinase (MAPK) *in vivo*. Fosforylering (synoniem voor activatie) van ERK-1/2, JNK, c-Jun, Elk-1 en ATF-2 wordt waargenomen binnen 60 minuten in de groep beademd met drukken die overrekking veroorzaken, in tegenstelling tot de normaal- en niet-beademde ratten. Verder wordt de transcriptie factor NF-κB cascade geactiveerd door beademing met drukken die overrekking veroorzaken. Zowel MAPK als ook NF-κB activatie kunnen bijdragen aan pulmonale inflammatie en proliferatie.

In **Hoofdstuk 6** verlengen we de overrekking waaraan een gezonde long wordt blootgesteld door de hoeveelheid surfactant tot supra-fysiologisch niveau te verhogen. Tijdens mechanische beademing met drukken die overrekking veroorzaken, wordt actief surfactant uit de distale luchtwegen geperst, wat verlaging van de rek op het longweefsel geeft bij onveranderde beademingsdrukken. In de studie beschreven in Hoofdstuk 6 wordt een additionele hoeveelheid actief surfactant in de long aangebracht om het fenomeen van surfactant verlies naar de distale luchtwegen te compenseren. Zoals waargenomen in Hoofdstuk 5 zorgde de hoge constante beademingsdrukken voor vergelijkbare activatie van de MAPK cascade. Ook MIP-2 en IL-6 werden uitgescheiden in de longen die beademd werden met hoge drukken die overrekking veroorzaken, gelijk aan de waarnemingen in Hoofdstuk 4. Het door surfactant verlengen van de rekbaarheid in een normale long gaf geen verdere significante activatie van de MAPK cascade of productie van MIP-2 of IL-6. Echter het verlengen van de rekbaarheid met exogeen surfactant resulteerde wel in verhoogde afgifte van TNF-α.

In **Hoofdstuk 7** wordt verhoogde expressie van de cytokine mRNA waargenomen tijdens schadelijke mechanische beademing van een geïnflammeerde long. Heat shock proteins (HSP) worden geproduceerd door cellen die blootgesteld worden aan diverse schadelijke stimuli (hypoxie/hitte. In deze studie laten wij zien dat ook schadelijke beademing HSP-productie kan activeren.

Amato en collega's hebben bewezen dat een longbeschermende beademingsstrategie die een actieve recruitment procedure combineert met een PEEP niveau dat het nieuw gerekruteerde longweefsel stabiliseert, gecombineerd met zo laag mogelijke slagvolumes, de mortaliteit in ARDS patiënten vermindert. In **Hoofdstuk 8** vergelijken we het effect van mechanische beademing op afgifte van ontstekingsmediatoren in surfactant deficiënt gemaakte biggen, tijdens beademing met een zelfde long beschermende beademingsstrategie (het Open Long Concept) of met een conventioneel beademingsstrategie. Beademing met het Open Long Concept verminderde de influx van neutrofielen in de long en verlaagde de hoeveelheid IL-8 in de long. Tenslotte was de thrombine activiteit ook significant lager in de Open Long Concept groep.

In de studie beschreven in **Hoofdstuk 9** laten we zien dat een longbeschermende beademingsvorm de bacteriegroei remt en disseminatie van bacteriën naar het bloed vermindert. *Klebsiella pneumoniae* infectie verhoogt het inflammatieniveau in de long en verhoogt de accumulatie van neutrofielen. Wanneer het endogene surfactant uit de long gespoeld is, versnelt dit de bacteriegroei. De verlaagde hoeveelheid surfactant verandert ook de permeabiliteit van het alveolaire-capillaire membraan, wat leidt tot een toename van het aantal positieve bloedkweken. De barrière functie kon echter behouden worden wanneer een long beschermende beademingsstrategie werd toegepast.

Concluderend: de studies beschreven in dit proefschrift benadrukken de essentiële invloed die mechanische beademing uitoefent op de progressie van de immuunreactie in een long door op diverse niveaus,variërend van rek-geïnduceerde activatie van intracellulaire messenger eiwitten zoals MAPK en NF-κB tot de activatie van mRNA en de uiteindelijke afgifte van diverse inflammatoire cytokines. Daarbij verstoort beademing de normale barrièrefunctie van de alveolaire-capillaire membraan hetgeen resulteert in verlies van compartimentalisatie van de cytokine respons. Tenslotte wordt zelfs bacteriegroei/kolonisatie direct beïnvloed door mechanische beademingsstrategie. Dus wanneer beademing noodzakelijk is, en zeker in reeds

beschadigde longen (ARDS), heeft de toegepaste beademingsstrategie een effect op de gehele inflammatie cascade en uiteindelijk ook op de uitkomst.

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Als eerste, de hiervolgende opsomming van mensen gaat niet in orde van belang maar in een soort 'Haitsma' logica.

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Prof. Dr. S. Uhlig, Dear Stefan. Since my Deutsch is as bad as your Nederland's, I will write this part in English. Stefan, thanks for being a part of my committee but even more for being part of a large part of the articles in this thesis. Through Prof. Lachmann, we started a very fruitful collaboration but it was not until we met at the infamous floating surfactant congress that I got to know you well. You were my roommate, drinking buddy and had the same lousy singing voice; I must still congratulate you for the hilarious anecdote, by returning my underwear by post to my university address. Thanks again and hopefully I can return the favour one day.

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De medewerkers van de afdelingen Cardiologie, Microbiologie en Kindergeneeskunde alhier, Immunologie in Utrecht, Inwendige Geneeskunde in Amsterdam and the Division of Pulmonary Pharmacology in Borstel, Germany.

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Papers

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CURRICULUM VITAE

Jack Haitsma was born on 20th September 1968, in Singapore. He graduated as a medical doctor in 1997 from the Erasmus University Rotterdam. He worked from 1997-1999 as a 'Verzekeringsarts' at the uitvoeringsinstelling GUO. Since 1999 he has been employed fulltime as a researcher at the Department of Anesthesiology, Erasmus University Rotterdam. From 1999-2002, Jack Haitsma was a PhD candidate at the Department of Anesthesiology under the supervision of Prof. Dr. B. Lachmann. He will continue working at the Department of Anesthesiology where his scientific focus will be the interaction of mechanical ventilation, multi-organ failure and sepsis, with special attention for the role of surfactant.

He has international collaboration with the Division of Pulmonary Pharmacology, Research Centre Borstel, Germany; Departments of Paediatric Pulmonology & Neonatology, Hannover Medical School, Hannover, Germany; and the Department of Clinical Physiology, Lund University, Malmö, Sweden.