Reduced activation of immunomodulatory transcription factors during positive end-expiratory pressure adjustment based on volume-dependent compliance in isolated perfused rabbit lungs

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Background. Repeated alveolar collapse and cyclic alveolar overdistension with associated activation of inflammatory signalling cascades contribute to ventilator-induced lung injury (VILI). The appropriate positive end-expiratory pressure (PEEP) which prevents or ameliorates VILI is unknown. In the isolated perfused lung, repeated adjustments of PEEP based on the continuously analysed intratidal compliance–volume curve have previously been shown to result in full end-expiratory alveolar recruitment and low risk of cyclic alveolar overdistension. Accordingly, we tested the hypothesis that such ventilatory management reduces intrapulmonary activation of the immunomodulatory transcription factors nuclear factor kB (NF-kB), activator protein 1 (AP-1) and cAMP-responsive element binding protein (CREB) which induce the expression of various chemokines and cytokines.

Methods. Isolated perfused rabbit lungs were randomly allocated to one of three groups: zero end-expiratory pressure (ZEEP) to induce repeated alveolar collapse (n=6), high PEEP to induce cyclic alveolar overdistension (n=6) and repeated PEEP adjustments based on intratidal compliance–volume curve analysis by the slice method to minimize repeated alveolar collapse and overdistension (n=9). All lungs were ventilated with a tidal volume of 6 ml kg⁻¹ bodyweight for 120 min. Thereafter, activation of transcription factors NF-kB, AP-1 and CREB in lung tissue was analysed by electrophoretic mobility shift assay.

Results. High PEEP was associated with the highest activation of NF-kB and AP-1 and repeated PEEP adjustments with the lowest activation when compared with the other two study groups (P<0.001). In contrast, activation of CREB did not differ between groups. Activated NF-kB and AP-1 protein complexes consisted mainly of the transactivators p50/p65 and c-Fos/Jun, respectively.

Conclusions. In isolated perfused rabbit lungs, repeated adjustments of PEEP based on the continuously analysed intratidal compliance–volume curve were associated with less activation of early steps of inflammatory signalling cascades than ventilation with ZEEP or high PEEP.

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Ventilation with either very low or very high positive end-expiratory pressure (PEEP) even during small tidal volume (V₁) ventilation causes ventilator-induced lung injury (VILI), suggesting that repeated alveolar collapse and cyclic alveolar overdistension are contributing factors. VILI is accompanied by pulmonary expression of cytokines and chemokines induced by cellular mechanical stress.²⁻⁴ The immunomodulatory transcription factors nuclear factor
kB (NF-κB), activator protein 1 (AP-1) and cAMP-responsive element binding protein (CREB) are involved in the expression of these mediators. Injurious mechanical ventilation activates NF-κB with consecutive induction of chemokine expression in pulmonary cells and isolated lungs. Likewise, AP-1 and CREB are activated in pulmonary cells by cyclic stretch. However, such activation has neither been confirmed in intact lungs nor assessed during ventilation with small VT and different PEEP settings.

Use of PEEP is part of routine ventilatory management. Optimum PEEP may be defined as one which prevents repeated end-expiratory alveolar collapse without causing cyclic alveolar overdistension. Such repeated alveolar collapse is detectable in the isolated perfused rabbit lung by continuous analysis of the intratidal compliance–volume curve using the slice method. We recently demonstrated in the isolated perfused rabbit lung that repeated adjustments of PEEP based on the intratidal course of C_{slice} result in full end-expiratory alveolar recruitment and low risk of cyclic alveolar overdistension. However, the impact of this approach on pulmonary transcription factor activation remains to be defined. Accordingly, the aim of this study was to investigate the effect of this ventilatory management on early steps of pro-inflammatory signalling cascades in the lung. We hypothesized that intrapulmonary activation of the immunomodulatory transcription factors NF-κB, AP-1 and CREB, which induce the expression of various chemokines and cytokines, would be reduced by the new ventilatory approach.

Materials and methods

Preparation of isolated perfused rabbit lungs

The isolated perfused rabbit lung model has been described in detail previously. Briefly, following Internal Review Board approval, rabbits of both sexes weighing 1.8–3.3 kg were anaesthetized intravenously with a mixture of ketamine (Parke-Davis, Berlin, Germany) and xylazine (Bayer Vital, Leverkusen, Germany) and anticoagulated with i.v. heparin (1000 U kg⁻¹). After tracheostomy, the lungs were ventilated with a VT of 6 ml kg⁻¹, a PEEP of 2 cm H₂O (PEEP valve, VBM, Sulz, Germany) and warmed room air using a Harvard ventilator (Hugo Sachs Elektronik, March Hugstetten, Germany). Following mid-ternal thoracotomy, the lungs were perfused with Krebs–Henseleit hydroxyethylamyllopectine buffer (Serag-Wiessner, Naila, Germany) via catheters inserted into the pulmonary artery and the left atrium. Sterilized perfusion circuits were used in all experiments. To render the lungs free of blood, the perfusate was initially not recirculated. With the onset of perfusion, the inspired gas was supplemented with 5% carbon dioxide. The lungs were removed from the thorax without interrupting ventilation or perfusion and freely suspended in a humidified chamber at 37.5°C. After wash-out of blood, the perfusion rate was slowly increased in a recirculating system to 150 ml min⁻¹ (Masterflex, Cole Parmer, Vernon Hill, IL, USA). Pulmonary venous pressure was set at 1.0–1.5 mm Hg. Pulmonary artery and left atrial pressures were measured via small-diameter tubing threaded into the perfusion catheters and connected to pressure transducers (Medex, Ratingen, Germany) which were adjusted to zero pressure at the level of the hilum.

Measurement of pulmonary mechanics

A detailed description of measurements and analysis of respiratory mechanics is given elsewhere. Briefly, airway pressure was measured with a transducer (1210A, IC Sensors, Milpitas, CA, USA) at the site of a heated pneumotachograph (Fleisch No. 0, Metabo, Epalinges, Switzerland) connected to the endotracheal tube via a three-way stopcock (Discoflex-3, B. Braun, Melsungen, Germany). The signals acquired were digitized and data were processed online using a slightly modified slice method which automatically considers intrinsic PEEP. The slice method divides every VT into six equal portions (slices). One value of compliance and resistance is determined for each slice by multiple linear regression analysis. Volume-dependent compliance (C_{slice}) within VT and pressure–volume loops were continuously monitored.

Experimental protocol

After initial preparation, 30 lungs were randomly assigned to one of three groups (see below) by drawing lots. After a subsequent 45 min stabilization period, during which ventilation was maintained constant in all groups (VT=6 ml kg⁻¹, 10 ventilations per minute), and standardization of volume history (sustained inflation with 20 cm H₂O for 30 s), lungs were included in the study if the following criteria were met: homogeneous white appearance with no signs of haemostasis, oedema, or atelectasis, pulmonary artery and ventilation pressures in the normal range, no air leak (continuous online monitoring of pressure–volume loops) and no pH instability. By these criteria, 21 of the 30 initially randomized lungs were included in the following three groups.

(1) R-PEEP: repeated PEEP adjustment based on the course of the intratidal dynamic compliance (n=9). Starting from 2.0 cm H₂O, PEEP was increased every 3 min in 0.5 cm H₂O increments to the lowest PEEP resulting in a descending intratidal C_{slice} curve. This compliance pattern indicates full end-expiratory alveolar recruitment in the isolated perfused rabbit lung. If necessary, PEEP was readjusted every 15 min to maintain this compliance pattern.

(2) ZEEP: zero end-expiratory pressure (aimed at inducing repeated end-expiratory alveolar collapse) (n=6).

(3) H-PEEP: high PEEP titrated to achieve a plateau pressure (P_{plat}) of 20–25 cm H₂O (aimed at inducing...
end-inspiratory alveolar overdistension) \((n=6)\). Preliminary studies had shown that a \(P_{\text{plat}}\) slightly higher than 25 cm H\(_2\)O caused frequent air leaks. PEEP was readjusted every 15 min to maintain \(P_{\text{plat}}\) within the target range.

Two hours after the start of the respective ventilatory strategy, lungs were snap frozen, minced in liquid nitrogen and stored at \(-70^\circ\text{C}\).

Total protein extracts of lung tissue and electrophoretic mobility shift assay (EMSA)

Frozen lung samples were homogenized in high-salt detergent buffer [HEPES (pH 7.9) 20 mM, NaCl 350 mM], glycerol 20% (vol vol\(^{-1}\)), Nonidet P-40 1% (vol vol\(^{-1}\)), MgCl\(_2\) 1 mM, EDTA 0.5 mM, EGTA 0.1 mM, dithiothreitol 1 mM, aprotinin 10 \(\mu\)g ml\(^{-1}\), Leupeptin 25 \(\mu\)M, NaF 10 mM, phenylmethylsulphonylfluoride 0.1% and phosphatase inhibitor cocktail 10 \(\mu\)l ml\(^{-1}\) (Calbiochem, La Jolla, CA, USA). Preparation of whole-cell extracts and EMSA for NF-\(\kappa\)B, AP-1 and CREB were performed as previously described.\(^{15,16}\) Equal amounts of total cellular protein (10–25 \(\mu\)g) were used for incubation with \([\gamma^{32}\text{P}]\)-labelled NF-\(\kappa\)B, AP-1, and CREB oligonucleotides. To demonstrate specific binding of oligonucleotides, the binding reactions were co-incubated with a 50-fold molar excess of either unlabelled specific or non-specific competitor. DNA binding proteins were identified by supershift experiments. Reaction mixtures were co-incubated with antibodies against the respective transcription factor (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA). After gel electrophoresis, gels were dried and specific signals were quantified using a phosphorimager system (FLA 2000, Raytest, Straubenhardt, Germany), analysed with AIDA software (version 3.10, raytest, Straubenhardt, Germany) and subsequently exposed to X-ray films with an intensifying screen at \(-70^\circ\text{C}\).

Data analysis

EMSA, PEEP and \(P_{\text{plat}}\) data were analysed by one-way ANOVA or two-way ANOVA for repeated measurements, where appropriate, followed by the Tukey test \((P<0.05)\) using the SigmaStat software package (version 2.03, SPSS, Chicago, IL, USA). Data are presented as medians (IQR) or means (SD).

Results

Effect of different PEEP settings on the course of \(C_{\text{slice}}\)

The plots of \(C_{\text{slice}}\) vs \(V_T\) indicate full end-expiratory alveolar recruitment during R-PEEP (descending \(C_{\text{slice}}\)), ongoing intratidal alveolar recruitment during ZEEP (bow-like \(C_{\text{slice}}\)) and cyclic alveolar overdistension during H-PEEP (flat \(C_{\text{slice}}\)) (Fig. 1).\(^{13}\)

Following the initial stabilization period, PEEP had to be increased \(~2\)-fold in the R-PEEP group and \(~3\)-fold in the H-PEEP group according to the respective protocols (Table 1). During the 120 min investigation period, \(P_{\text{plat}}\) increased by \(~25\%\) in the R-PEEP group but by \(~250\%\) in the ZEEP group, indicating a much greater decrease in compliance in the latter (Table 1).
Effect of different PEEP settings on the pulmonary activation of NF-κB, AP-1 and CREB

NF-κB, AP-1 and CREB were activated after 120 min of ventilation in all groups (Fig. 2A and B). R-PEEP was associated with a significantly lower activity ($P<0.001$) of NF-κB and AP-1 (Fig. 2A and B) than ZEEP or H-PEEP. In contrast, CREB activation did not differ among the groups (Fig. 2C).

The activated NF-κB complex consisted of p50/p50 homodimers and p50/p65 heterodimers but did not involve cRel proteins. The AP-1 DNA binding complex was composed of c-Fos and c-Jun. DNA-binding CREB proteins involved CREB1 and CREB2. The amount of activated p50/p65 and p50/p50 complexes was higher during ZEEP.

Table 1  PEEP and plateau airway pressures obtained from isolated perfused ventilated rabbit lungs at the end of the stabilization period, at 0 min after initial adjustments and after 120 min (at the end of the investigation period) for three different interventions: R-PEEP, repeated PEEP adjustment based on analysis of the intratidal course of volume-dependent compliance; ZEEP, zero end-expiratory pressure; H-PEEP, PEEP resulting in $P_{plat}=20–25$ cm H$_2$O. Data are presented as means (sd); $^*P<0.001$ vs end of stabilization; $^\dagger P<0.002$ vs 0 min

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<th>R-PEEP</th>
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<tr>
<td><strong>PEEP (cm H$_2$O)</strong></td>
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<td>End of stabilization</td>
<td>2.0 (0.0)</td>
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<td>0 min</td>
<td>3.7 (0.5)$^*$</td>
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<td>6.3 (0.5)$^*$</td>
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<td>120 min</td>
<td>4.5 (0.9)</td>
<td>0.0 (0.0)</td>
<td>7.5 (0.4)</td>
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<td><strong>$P_{plat}$ (cm H$_2$O)</strong></td>
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<td>End of stabilization</td>
<td>4.4 (0.4)</td>
<td>4.1 (0.4)</td>
<td>4.3 (0.4)</td>
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<tr>
<td>0 min</td>
<td>8.5 (1.5)$^*$</td>
<td>4.6 (1.3)</td>
<td>21.5 (1.4)</td>
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<td>120 min</td>
<td>10.7 (1.8)$^\dagger$</td>
<td>12.1 (1.5)$^\dagger$</td>
<td>21.0 (0.8)</td>
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Fig 2  Left panels: representative EMSA of (A) NF-κB, (B) AP-1 and (C) CREB with protein extracts of isolated perfused rabbit lungs ventilated for 120 min with different regimens. R-PEEP, repeated PEEP adjustments with regard to the intratidal compliance aiming at full alveolar recruitment; ZEEP, zero end-expiratory pressure; H-PEEP, high PEEP causing $P_{plat}=20–25$ cm H$_2$O. Right panels: transcription factor activation of all lungs in arbitrary phosphorimager units: data are presented as medians (IQR). PSL, photostimulated luminescence. $^*P<0.001$ vs R-PEEP; $^\dagger P<0.001$ vs ZEEP.
and H-PEEP than during R-PEEP (Fig. 3). The amount of activated p50/p65 complexes was 3-, 4- and 10-fold greater than the amount of activated p50/p50 during R-PEEP, ZEEP and H-PEEP, respectively (Fig. 3).

**Discussion**

In this study, repeated adjustments of PEEP based on the continuously analysed intratidal compliance–volume curve were associated with less NF-κB and AP-1 activation in lung tissue than the application of zero or very high PEEP. We have recently shown that, in contrast with a fixed PEEP setting, adjustment of PEEP based on the intratidal compliance–volume curve prevented end-expiratory alveolar de-recruitment in isolated perfused rabbit lungs.13 This ventilatory strategy consists of repeated titration of PEEP to the lowest level at which the intratidal volume-dependent compliance curve elicits a descending character (Fig. 1A), as assessed by the slice method (C\text{slice}).12

A relationship between intratidal pulmonary mechanics and the pulmonary inflammatory response has previously been demonstrated during constant-flow ventilation.17 18 In contrast with our study, ventilation was adjusted on the basis of the inspiratory pressure–time curve (stress index).17 The algorithm of the slice method used in the present study analyses both inspiratory and expiratory changes of the compliance–volume relationship during non-constant flow conditions.12

**In vitro**, cyclic mechanical stress apparently causes an inflammatory response predominantly in human lung macrophages but also in alveolar type II like epithelial cells and bronchial epithelial cells.8 10 A similar inflammatory response can be induced in isolated lungs during artificial ventilation: Whereas previous investigators concentrated on pro-inflammatory mediator expression,21 27 we focused on the early steps of the pro-inflammatory signalling cascades. Activation of NF-κB and AP-1 represents a crucial early event during activation of inflammatory signalling pathways in pulmonary cells.19 20 Our finding of activation of these transcription factors by ventilation with high PEEP is consistent with previous studies showing activation by cyclic stretch in pulmonary cells8–10 and lung parenchyma.21 In accordance with findings in bronchial epithelial cells treated with cyclic stretch,10 we detected CREB activation in whole-lung tissue. It is not clear why CREB activation in isolated lungs did not differ between study groups. **In vitro**, CREB activation caused by cyclic mechanical stress seems to follow a different course than activation of NF-κB and AP-1.10 22 Therefore we cannot exclude the possibility that ventilation with different PEEP settings might have caused a difference in CREB activation before or after the 2 h observation point of our study. Pulmonary activation of NF-κB during ventilation with large tidal volumes has previously been reported in isolated lungs,3 but neither activation of NF-κB during ventilation with small tidal volumes nor activation of CREB and AP-1 during mechanical ventilation in general have been reported. AP-1 is comprised of homodimeric and heterodimeric complexes of members of the Jun and Fos families and constitutes an important regulator of cell proliferation and related events. Moreover, there is increasing evidence that activation of AP-1 plays a pivotal role in the pro-inflammatory signalling pathways of acute lung injury.19 21 23 AP-1 activation leads to IL-8 transcription8 which, in turn, is responsible for the chemoattraction of neutrophils to the lung, a critical step in the development of VILI.24

**Limitations**

There are several limitations to this study. Data were obtained in the isolated perfused rabbit lung. For obvious reasons, this model does not reflect clinical reality. Nevertheless, it represents an accepted model in the study of VILI-related problems.13 18 25–27 It remains to be determined whether a reduction of repeated end-expiratory alveolar collapse and cyclic alveolar overdistension are the main mechanisms by which R-PEEP resulted in less activation of the transcription factors than ZEEP and H-PEEP. The degree of correlation between early intrapulmonary activation of the transcription factors on the one hand and indices of lung injury (e.g. oxygenation, histology) and other inflammatory responses (e.g. broncho-alveolar release of cytokines, PMN infiltration) on the other remains to be defined. Clearly, the observed activation of transcription factors cannot be equated with cytokine expression. However, ventilation with high PEEP induced the NF-κB heterodimer p50/p65 and the AP-1 heterodimer c-Fos/Jun which are strong transactivators of gene transcription.5 28 The activation of NF-κB and AP-1 during ventilation with ZEEP observed in our model is in accordance with the induction of pro-inflammatory cytokines observed in other recent experimental studies.4 29 30 Nevertheless, direct translation of the results into clinical practice is unwarranted.
Conclusions
During small tidal volume ventilation in an isolated perfused rabbit lung model, repeated adjustments of PEEP based on the intratidal course of volume-dependent compliance were associated with less activation of the immunomodulatory transcription factors NF-κB and AP-1 than ventilation with ZEEP or high PEEP. These findings are consistent with our hypothesis that a ventilatory strategy aimed at preventing repeated end-expiratory alveolar collapse and cyclic alveolar overdistension reduces the activation of inflammatory signalling cascades in the lung at an early stage. The impact of this ventilatory strategy on clinical practice remains to be determined.

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During the course of this work Gerd Hermle died unexpectedly. We have lost a skilled scientist, a dedicated physician and a close friend.

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