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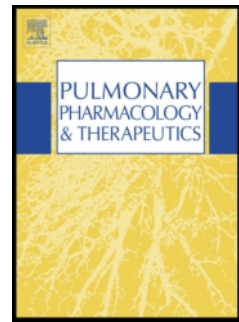
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Selective NF- κ B inhibition, but not dexamethasone, decreases acute lung injury in a newborn piglet airway inflammation model

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Abstract

Acute respiratory failure in neonates (e.g., ARDS, meconium aspiration pneumonitis, pneumonia) is characterized by an excessive inflammatory response, governing the migration of polymorpho-nuclear leukocytes (PMNL) into lung tissue and causing consecutive impairment of gas exchange and lung function. Critical to this inflammatory response is the activation of nuclear factor- κ B (NF- κ B) that is required for transcription of the genes for many pro-inflammatory mediators. We asked whether the inhibition of NF- κ B activity using either a selective inhibitor (IKK-NBD peptide) or dexamethasone would be more effective in decreasing NF- κ B activity and chemokine expression in pulmonary cells. Changes in lung function were repeatedly assessed for 24h following induction of acute respiratory failure and therapeutic intervention.

We conducted a randomized, controlled, prospective animal study with mechanically ventilated newborn piglets which underwent repeated airway lavage (20 ± 2 [SEM]) to remove surfactant and to induce lung inflammation. Admixed to 100 mg kg^{-1} surfactant, piglets then received either IKK-NBD peptide (S+IKK), a selective inhibitor of NF- κ B activation, its control peptide without intrinsic activity, dexamethasone (S+Dexa), its solvent aqua, or an air bolus only (all groups $n=8$).

After 24h of mechanical ventilation, the following differences were measured: $\text{PaO}_2/\text{FiO}_2$ (S+IKK 230 ± 9 mmHg vs. S+Dexa 188 ± 14 , $p < 0.05$); ventilation efficiency index (0.18 ± 0.01 [$3800/(\text{PIP}-\text{PEEP}) \cdot f \cdot \text{PaCO}_2$] vs. 0.14 ± 0.01 , $p < 0.05$); extravascular lung water ($24 \pm 1 \text{ ml kg}^{-1}$ vs. 29 ± 2 , $p < 0.05$); PMNL in BAL fluid ($112 \pm 21 \text{ cells } \mu\text{l}^{-1}$ vs. 208 ± 34 , $p < 0.05$), IL-8 ($351 \pm 117 \text{ pg ml}^{-1}$ vs. 491 ± 144 , $p = \text{ns}$) and leukotriene B_4 ($23 \pm 7 \text{ pg ml}^{-1}$ vs. 71 ± 11 , $p < 0.01$) in BAL fluid. NF- κ B activity in the nucleus of pulmonary cells differed by $32 \pm 5 \%$ vs. 55 ± 3 , $p < 0.001$. Differences between these two intervention groups were more pronounced in the second half of the observation period (hours 12-24).

At 24h of mechanical ventilation, inhibition of NF- κ B activity by IKK-NBD peptide admixed to surfactant as a carrier caused improved gas exchange, lung function and reduced pulmonary inflammation, as evidenced by reduction in PMNL migration into lung tissue due to reduced nuclear NF- κ B activity. We conclude that IKK-NBD admixture to surfactant in acute neonatal respiratory failure is superior to dexamethasone administration within the first 24h.

Keywords

Lung inflammation; nuclear factor- κ B; polymorpho-nuclear leukocytes; chemokines; dexamethasone; IKK-NBD peptide; acute lung injury; respiratory failure; newborn piglets

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1. Introduction

In neonatal inflammatory lung diseases with respiratory failure such as acute respiratory distress syndrome (ARDS), an increased expression of cytokines, selectins, integrins, and chemokines can be found to cause the invasion of inflammatory cells into lung tissue, which contribute to the degradation of surfactant films to hyaline membranes within the alveoli, and to the formation of a proteinaceous edema within the pulmonary interstitial space [1, 2]. Therefore, glucocorticoids have been used to alleviate respiratory failure due to the damage caused by polymorpho-nuclear leukocytes (PMNL) and alveolar macrophages in their metabolically activated states. They promote surfactant synthesis in type II pneumocytes [3], and inhibit the production of proinflammatory mediators through gene expression by activated glucocorticoid receptor binding to negative glucocorticoid response elements in the 5'-flanking region of proinflammatory cytokine genes [4], thereby depressing the activity of the nuclear factor kappa B (NF- κ B), a transcription factor that is required for transcription of the genes for many pro-inflammatory mediators, including adhesion molecules, cytokines, and chemokines [5, 6].

However, a recent large clinical trial on adult ARDS patients could not demonstrate a decreased mortality with the use of systemic methylprednisolone in the hyperinflammatory phase [7].

In contrast, animal trials of different types of acute inflammatory lung injury using surfactant [8] or liposomes [9, 10] as carrier substances for dexamethasone showed positive results, as the overall glucocorticoid dose related to body weight was reduced and homogenous distribution of the drug within the lung was achieved. The use of intratracheal dexamethasone without a lipophilic vehicle substance and involving mechanical ventilation yielded only minor positive effects for gas exchange [11], and PMNL concentrations in broncho-alveolar lavage fluid (BALF) [12] unless extremely high doses (5 mg/kg) were administered [13].

The aim of this study was to compare the short term effects of intratracheally administered IKK-NBD peptide (a selective cell permeable oligopeptide that selectively inhibits the NF- κ B activation; see chapter 2.3) with dexamethasone on lung function in a newborn inflammatory model of acute lung injury induced by repeated airway lavage. The drugs were admixed to surfactant that was used as a carrier substance. The rationale for this comparison was the assumption that selective NF- κ B inhibition might act faster than dexamethasone and would exert its effects within 24h of mechanical ventilation, then being a reasonable choice of treatment in a setting of acute respiratory failure. The piglets were

ventilated for 24 h while gas exchange, lung mechanics, lung volumes, and extravascular lung water (EVLW) were repeatedly determined, followed by *ex vivo* analyses of BALF for PMNL and mononuclear cells, chemokine concentrations, and NF- κ B activity in pulmonary cells.

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2. Materials and Methods

2.1 Animal preparation and clinical care

The experimental protocol was approved by the local review board for the care of animal subjects in accordance with the German law on animal protection (BGBl 1, page 1319). 40 male piglets (mixed country breed) weighing 2.2 ± 0.1 [SEM] kg were studied in pairs of two between days 2 and 5 of age.

Initially 0.025 mg/kg atropine, 10 mg/kg ketamine and 1 mg/kg midazolam hydrochloride were administered to allow oral intubation with an uncuffed 3.5 mm endotracheal tube (ETT). Anesthesia and muscle paralysis were maintained by continuous infusion of 16 mg/kg propofol and 0.17 mg/kg pancuronium bromide per hour. To prevent leakage the tube was tightly secured in place by a peritracheal ligature. A thermodilution catheter was inserted into the left femoral artery for monitoring of hemodynamic parameters and arterial blood gases. The right internal jugular vein was catheterized for infusion therapy and for cold bolus injection for performing thermodilution measurements.

2.2 Mechanical Ventilation and Airway Lavage

Mechanical ventilation was provided by two time-cycled pressure-limited infant ventilators (Babylog 1, Dräger, Germany) at $FiO_2 = 0.5$, positive end-expiratory pressure (PEEP) = 0.6 kPa, flow = 8 l min^{-1} , inspiratory time = 0.4 s, ventilator rate = 25 min^{-1} before lavage and 35 min^{-1} during the sequence of repeated airway lavage until intervention. Peak inspiratory pressure (PIP) was adjusted every hour to keep tidal volume (V_T) at 7 ml kg^{-1} .

Repeated airway lavage was carried out by the instillation and removal of 30 ml kg^{-1} of warmed normal saline via the ETT over a 30s period. Airway lavage was then repeated every 3-5 min until both the PaO_2/FiO_2 decreased to $\sim 85 \text{ mmHg}$, and a $PIP \geq 2.0 \text{ kPa}$ was required to maintain V_T at 7 ml kg^{-1} . To maintain $PaCO_2$ within a range of 35-50 mmHg, the ventilator rate was changed according to the following protocol: reduced by -5 min^{-1} , if $PaCO_2 < 35 \text{ mmHg}$; reduced by -10 min^{-1} , if $PaCO_2 < 30 \text{ mmHg}$; increased by $+5 \text{ min}^{-1}$, if $PaCO_2 > 50 \text{ mmHg}$; increased by $+10 \text{ min}^{-1}$, if $PaCO_2 > 60 \text{ mmHg}$. FiO_2 was adjusted every hour to stay within the limits for PaO_2 of >40 to $<150 \text{ mmHg}$ using the following protocol: FiO_2 increased by 0.1, if $PaO_2 < 40 \text{ mmHg}$; FiO_2 reduced by 0.05, if $PaO_2 > 150 \text{ mmHg}$. Besides surfactant depletion and iatrogenic pulmonary edema, repeated airway lavage acts as a potent pro-inflammatory stimulus causing the expression of chemokines and the migration of PMNL and mononuclear cells in their metabolically activated state into lung parenchyma, even to an extent that peripheral neutropenia is reached [14].

2.3 Experimental protocol, randomization, surfactant and drugs

Gas exchange parameters (PaO_2 and PaCO_2) were measured every 1-2 hours; lung function parameters (functional residual capacity (FRC), dynamic compliance of the respiratory system (C_{rs}), alveolar portion of the tidal volume (V_A), and tidal volume (V_T)) and hemodynamic parameters (notably extravascular lung water (EVLW)) were measured before airway lavage, after airway lavage at baseline and at 1h, 6h, 12h, 18h and 24h after intervention.

At baseline the animals were randomized in pairs of two ($n = 8$ per group) to minimize differences in physiology caused by age, weight and litter. Prepared combinations for randomization were, as defined below: either piglet 1 S+IKK / piglet 2 S+CP, or piglet 1 S+CP / piglet 2 S+IKK, or piglet 1 S+Dexa / piglet 2 S+Aqua, or piglet 1 S+Aqua / piglet 2 S+Dexa, or piglet 1 C / piglet 2 C.

The surfactant preparation HL-10 (Halas Pharma BV, Rotterdam, The Netherlands) was administered at a dose of 100 mg kg^{-1} at 2.5 ml kg^{-1} . It is a freeze-dried surfactant isolated from minced porcine lungs and consists of approximately 90% phospholipids, 0.6% surfactant protein B, and 0.4% surfactant protein C.

In the S+IKK group (group 1), 1.25 mg per piglet (i.e. $568 \text{ } \mu\text{g kg}^{-1}$ as an average) of a selective NF- κ B inhibitor (IKK-NBD peptide, Biomol, Hamburg, Germany) was dissolved in $50 \text{ } \mu\text{l}$ DMSO 0.125% and was added to the surfactant preparation by gentle stirring. IKK-NBD peptide is a selective cell-permeable inhibitor of NF- κ B activation. It blocks the interaction of NEMO (NF- κ B *Essential Modulator*, an $\text{I}\kappa\text{B}\alpha$ complex regulatory protein) with the $\text{I}\kappa\text{B}$ kinase complex. The synthesis of the binding molecule $\text{I}\kappa\text{B}\alpha$ (I=inhibitor) is the key event in stopping NF- κ B activity, however, the rate-limiting step in activation of NF- κ B appears to lie in the activity of IKK [15]. IKK-NBD peptide (NBD stands for *NEMO Binding Domain*) is an inhibitor of NF- κ B activation which selectively blocks the interaction of NEMO with the IKK complex [16]. The peptide consists of 28 amino acids and forms two domains, an antenpedia homeodomain, which confers cell permeability, and the T735 to E745 region of $\text{IKK}\beta$, which is the NEMO binding domain (NBD) [17].

The S+CP group (group 2) piglets were treated with surfactant and an IKK-NBD control peptide (Biomol) which is structurally similar but rendered biologically inactive by replacing tryptophan with alanine in positions 22 and 24. Dexamethasone was purchased from Sigma-Aldrich (München, Germany). 0.25 mg per piglet (i.e. $108 \text{ } \mu\text{g kg}^{-1}$ as an average) was dissolved in 1 ml aqua and mixed with the surfactant preparation (S+Dexa, group 3).

Dexamethasone inhibits NF- κ B translocation, but also plays an integral role in stimulating surfactant synthesis through activation of the rate-regulatory enzyme for phosphatidylcholine synthesis: the cholinephosphate cytidylyltransferase [18, 3]. S+Aqua group (group 4) piglets received 1 ml aqua mixed with surfactant, as a control. Another control group (C, group 5) received 2.5 ml kg⁻¹ air into a second lumen of the ETT.

Surface tension-increasing properties of IKK-NBD peptide and dexamethasone as well of their solvents DMSO and aqua at their defined doses and dissolved in surfactant could be excluded by minimum and maximum surface tension measurements of 100 μ l of the surfactant preparations on the saline filled trough of a modified Wilhelmy balance (E. Biegler, Mauerbach, Austria), as previously described [19] (data not given).

2.4 Measurement of gas exchange, haemodynamics and lung function

PaO₂ and PaCO₂ were measured in blood samples taken from the femoral arterial catheter. PaCO₂ was used for calculating a ventilation efficiency index (VEI = [3800/(PIP-PEEP) * f * PaCO₂]) according to Notter et al. [20].

Cardiac output monitoring (PC 8000 PiCCO monitor, Pulsion, München, Germany) was performed using the transpulmonary indicator dilution technique (cold bolus of 2 ml normal saline <8°C), as previously described [21].

To calculate FRC, C_{rs}, V_A, and V_T, we used the nitrogen washout technique for lung volumes and the single breath least squares method for lung mechanics, as previously described [22].

2.5 Bronchoalveolar lavage fluid (BALF) and blood cell counts

Two lavages were performed for diagnostic purposes: the initial lavage in the sequence of lavages to establish lung injury, and a lavage after 24h of mechanical ventilation immediately before killing the piglets. BALF was filtered to remove gross particles and secretions from the airways, and was centrifuged for 4 min at 4°C and 2500 rev min⁻¹ to separate cells and cellular debris from the surfactant and lavage fluid.

2.6 Clinical care

A heating pad was used to maintain a constant core temperature in the piglets of 38 to 39°C. Each piglet received an infusion of D5W in 1/2 normal saline at a fluid intake of 80 ml kg⁻¹ d⁻¹. Two hours after intervention, 50 mg kg⁻¹ of Ampicillin/Sulbactam was given intravenously to prevent bacterial infections.

2.7 Interleukin-8 (IL-8) and leukotriene B₄ (LTB₄) assays

IL-8 concentrations in BALF were determined using the QuantikineP porcine IL-8/CXCL8 immunoassay (R&D Systems, Wiesbaden, Germany). LTB₄ concentrations were also measured by immunoassay (R&D Systems) which is based on the competition between LTB₄ and the LTB₄-alkaline phosphate conjugate for a limited amount of LTB₄ antiserum.

2.8 *Ex vivo* lung processing

After killing the piglets with an overdose of 1-molar KCl in deep sedation, the lungs were removed from the thorax, and all blood was cleansed from the pulmonary circulation by infusing normal saline into the main pulmonary artery. Small specimens of tissue from a ventral (non-dependent) position of the left lower lobe were taken to determine wet-dry-ratios by drying overnight at 60°C to remove any fluid.

2.9 NF- κ B activity in pulmonary cells

Preparation of cytosolic and nuclear extracts: Frozen lung tissue samples were pulverized and melted. The pulverized material was incubated in 10 mM TRIS, 5 mM MgCl₂, 10 mM KCl, 1 mM EGTA, and 10% Sucrose for 30 min at pH 7.4 and 4°C. Then, 10% NP-40 was added (Vol. 10/1). Samples were centrifuged for 1 min at 4°C and 16.000 x g, and 20 μ g of supernatant protein was subjected to Western blot analysis of cytosolic proteins. To obtain nuclear extracts, the pellets were resuspended for in 20 mM TRIS, 5 mM MgCl₂, 320 mM KCl, 0,2 mMol EGTA, and 25% Glycerol and incubated at pH 7.4 for 15 min at 4°C for nuclear lysis. Samples were centrifuged for 5 min at 4°C and 16.000 x g, and 20 μ g supernatant protein was analyzed by Western blot for nuclear proteins.

Western Blot Analysis: Equal amounts of protein were separated on 12.5% SDS-PAGE and electroblotted onto nitrocellulose membranes for 1h at 100 V and 4°C. Blots were blocked in 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBST) for 1h at room temperature and probed with anti NF- κ B p105 / p50 (AB # 3035, Cell Signaling Technology, Boston, USA), in the same buffer. After washing three times with TBST, blots were incubated with anti-rabbit IgG, HRP-linked as secondary antibodies (AB #7074, Cell Signaling Technology) for 1h at room temperature. Protein bands were visualized by enhanced chemoluminescence using Super Signal (GE Healthcare, München, Germany).

2.10 Statistical methods

We used a one-way analysis of variance to assess differences between the five groups for parameters measured at a single time point. Repeated-measures two-way analysis of

variance (ANOVA) was used to assess differences between groups in changes over time, thereby separating the first (1-12h) and the second (12-24h) half of the experimental period. An unpaired t-test was used for direct comparisons of S+IKK vs. S+Dexa.

Mean values are expressed \pm 1SEM. Differences in values of $p < 0.05$ were considered to be significant.

3. Results

3.1 Comparability of study groups

A total of 40 piglets weighing 2.2 ± 0.1 kg was studied in pairs of two according to a randomization protocol after airway lavage. One piglet was excluded from the C group because of insufficient surfactant removal from the airways due to a left bronchial dynamic stenosis. There were no significant differences between the five groups with regard to body weight, the number of lavages performed, loss of lavage fluid in the airways, final $\text{PaO}_2/\text{FiO}_2$ and PIP (the latter two parameters were also used as the endpoints for the lavage procedure) (Table 1).

3.2 Clinical parameters

$\text{PaO}_2/\text{FiO}_2$ levels (Figure 1) were superior in the S+IKK group (when compared to S+Dexa) only in the second half of the observational period (group difference $p < 0.05$, time factor $p = \text{ns}$); at 24h the following results were obtained: S+IKK 230 ± 9 mmHg vs. S+Dexa 188 ± 14 , $p < 0.05$ (unpaired t-test). VEI (Figure 2) differed between the two intervention groups within both time periods (1-12h: group difference $p < 0.01$, time factor $p < 0.001$; 12-24h: group difference $p < 0.001$, time factor $p = \text{ns}$). At 24h the following results were obtained: S+IKK 0.18 ± 0.01 [$3800/(\text{PIP}-\text{PEEP}) * f * \text{PaCO}_2$] vs. S+Dexa 0.14 ± 0.01 , $p < 0.05$.

Table 2 shows baseline, 1, 12, and 24h data for lung function parameters (FRC , C_{rs} , and V_A) and EVLW. While there were no statistical differences between the five groups at baseline (one-way ANOVA), at 24h the following differences were observed: FRC $p < 0.01$; C_{rs} $p < 0.05$; V_A $p < 0.0001$; and EVLW $p < 0.05$ (unpaired t-test). Treatment effects differed for FRC , C_{rs} , and V_A in both time periods; however, EVLW started to differ between groups not before 24h.

3.3 Leukocytes in blood and BALF

The blood leukocyte counts did not show significant differences between the 5 groups (Table 3), with C group values moderately higher than in the other four groups. Bands differed between the two intervention groups ($p < 0.05$) without having any influence on the

percentage of PMNL in peripheral blood. Leukocyte counts from BALF (Figure 3) were comparable in the intact lung, but showed significant differences (S+IKK 139 ± 26 cells μl^{-1} , S+Dexa 257 ± 43 , $p < 0.05$, unpaired t-test) between the two intervention groups at 24h. The percentage of PMNL in BALF at 24h was: S+IKK $81 \pm 1\%$, S+CP 88 ± 1 , S+Dexa 85 ± 2 , S+Aqua 84 ± 2 , C 90 ± 1 , $p = 0.07$ (one-way ANOVA); direct comparison of the two intervention groups was not significant ($p = 0.18$, unpaired t-test). The same statistical results also apply to mononuclear cells: S+IKK $18 \pm 1\%$, S+CP 12 ± 1 , S+Dexa 14 ± 2 , S+Aqua 15 ± 2 , C 10 ± 1 .

3.4 Chemokines in BALF

IL-8 concentrations (Figure 4) at 24h differed significantly between the five groups ($p < 0.01$, one-way ANOVA); but comparison of the two intervention groups was not significant: S+IKK 351 ± 117 pg ml^{-1} vs. S+Dexa 491 ± 144 (unpaired t-test). In contrast, as treatment differences of LTB_4 concentrations were not significant ($p = \text{ns}$), comparison of the two intervention groups, however, showed the following differences: S+IKK 23 ± 7 pg ml^{-1} vs. S+Dexa 71 ± 11 , $p < 0.01$.

3.5 Wet lung weight / dry lung weight - ratio

Wet-dry-ratios from small lung specimens were: S+IKK 7.6 ± 0.2 , S+CP 8.4 ± 0.2 , S+Dexa 8.7 ± 0.2 , S+Aqua 8.7 ± 0.4 , C 9.2 ± 0.2 ($p < 0.05$, one-way ANOVA; S+IKK vs. S+Dexa $p < 0.01$, unpaired t-test).

3.6 NF- κ B activity in pulmonary cells

NF- κ B activity in the nucleus and cytosol of pulmonary cells differed significantly ($p < 0.001$; $p < 0.001$, one-way ANOVA; Figure 5); direct comparison of the two intervention groups yielded the following results: nucleus S+IKK $32 \pm 5\%$, S+Dexa 55 ± 3 ($p < 0.01$, unpaired t-test); cytosol S+IKK $205 \pm 2\%$, S+Dexa 170 ± 2 , $p = \text{ns}$.

Discussion

The encouraging results of a previous study with IKK-NBD peptide [23] - notably reductions in EVLW and in PMNL concentration in BALF -, prompted us to compare the immunosuppressive short term effects of IKK-NBD peptide with those of dexamethasone in a similar model, but with an even higher degree of lung edema (average EVLW for all piglets at baseline: 51 ± 4 ml kg^{-1} vs. 42 ± 5 in the previous study) and for a much longer observation period (24h vs. 6h). IKK-NBD peptide admixed to a porcine surfactant preparation and compared to the effects of dexamethasone in surfactant resulted in significantly improved gas exchange and lung function, probably due to lower chemokine liberation and a decreased PMNL migration into the lung. Taking into account the time slope of our study, clinical improvements using IKK-NBD treatment did not occur before 12h (exceptions: VEI, C_{rs} , V_A) after its administration, while most of the dexamethasone effects was equal to the effects of surfactant treatment alone. Most importantly, reduction in NF- κ B activity in the nucleus of pulmonary cells was convincingly low in the S+IKK group and differed significantly from the values obtained in S+Dexa group pulmonary cells.

Two editorials [24, 15] have proposed the suppression of inflammatory activity during the hyperinflammatory phase of acute lung injury by targeting NF- κ B. NF- κ B seems to be a logical choice for investigation, as it is at the center of the signal transduction network and governs the expression of pro-inflammatory cytokines and chemokines and of pro-apoptotic factors. This is even more the case in neonatal pulmonary diseases, because studies with newborn PMNL vs. adult PMNL showed that e.g. TNF- α induced NF- κ B activation was even more pronounced in newborn PMNL [25], which underlines the significance of this pro-inflammatory pathway in newborn organisms.

The most obvious explanation for improvements in lung function in our present study is the suppression of the chemokines IL-8 and LTB₄ (Figure 4) followed by decreased cell migration into the lung (Figure 3). Other authors found that after LPS stimulus, IKK-NBD peptide counteracts reduced expression of G-protein-coupled receptor kinase by chemokine receptor desensitization and lowers PMNL migratory response [26]. It also prevents the increased toll-like receptor2 mRNA expression after LPS challenge [27]. In a mouse model, cyclooxygenase-2 levels and expression of TNF- α were reduced at a dose of 500 μg per mouse (i.e. ~ 20 mg kg^{-1}) together with a reduction in carrageenan induced paw edema [28].

IKK-NBD peptides from other manufacturers were shown to prevent LPS-induced CD11b protein expression and PMNL adhesion [29]. These experiments differ from ours, as IKK-NBD peptide administrations were carried out in adult animals and via the systemic route. The attenuation of PMNL migration into lung tissue is probably more effective using the systemic approach; however, systemic side effects might also occur more often in the delicate neonatal organism.

. Dexamethasone, however, did not exert any improvements in PaO₂/FiO₂, VEI or lung inflammation, when compared to the administration of Surfactant alone. This is explained by the fact, that nuclear NF-κB activity was not suppressed after 24h. As there are no clinical trials on treatment of newborn or pediatric ARDS with corticosteroids, adult animal models with intratracheal administration must be taken as surrogates: different authors have described attenuation of PMNL concentrations in BALF in an oleic-acid rat model [13, 12]; mild improvements in gas exchange and plasma malonyl dialdehyde in a hydrochloric acid rabbit model [11]; and improvements in lung compliance in an oleic-acid rat model [12].

Taken together, lung function was better preserved by IKK-NBD peptide than by dexamethasone as expressed by significant improvements in PaO₂/FiO₂ and VEI. A requisite for this “surfactant fortifying” effect is decreased PMNL migration into lung parenchyma [30] and attenuation of alveolar macrophages [31] (not tested in this study), and capillary leakage and transmission of large molecule peptides into lung tissue [32]. The approach of making exogenous surfactant less vulnerable to inflammatory attack within the airways was successfully tried with substances that share the property of binding water, such as e.g. hyaluronan in a rat model of meconium injury [33], or *in vitro* by plamalogens, an alk-1-enyl-acyl subclass of phosphatidylethanolamine and phosphatidylcholine [34].

Limitations: 1. Dosage: The IKK-NBD peptide dose of 568 μg kg⁻¹ dissolved in surfactant was derived from our previous experience in a similar model [23] when an average dose of 666 μg kg⁻¹ was chosen. May et al. [35] used a much larger dose of 200 μg per mouse (average body weight 25 g, i.e. approximately 8000 μg kg⁻¹) intraperitoneally to treat mechanical ear edema and zymosan-induced peritonitis. Lower doses may be equally effective, but a dose-response relationship has not yet been established. The average dexamethasone dose of 113 μg kg⁻¹ was adapted from clinical practice in the 80th and 90th with preterm infants developing BPD [36, 37] and single studies with term infants receiving

dexamethasone for inflammatory lung disease [38, 39] by reducing the systemic dose of 0.5 mg kg^{-1} in these studies by 80% for a topical dose. 2. Kinetics: There is little knowledge about how long the anti-inflammatory effect of IKK-NBD peptide lasts when given in a single dose. In contrast, in an adult rat model, free intratracheal dexamethasone peaks at 1h after its administration within the lung parenchyma while this peak is delayed to 4h by using liposomes as a vehicle [10]. 24h recovery of the drug is close to 0% of the initial dose given. This finding explains the observation of improved lung function in chronic lung disease of prematurity not before several doses of dexamethasone over a 72 hour interval [40]. 3. Drug interactions: Intermittent or permanent binding of some IKK-NBD peptide or its control peptide (both small molecules composed of 28 amino acids, molecular weight 3694 and 3464, respectively) or of dexamethasone (molecular weight 516) with surfactant components cannot be excluded. However, Nimmo et al. [8] proved unchanged surface tensions in surfactant films with dexamethasone admixed to the artificial surfactant Survanta[®]. 4. Solvents: As the IKK-NBD peptide / control peptide solvent DMSO (average dose 0.04 mg kg^{-1}) might induce NF- κ B activation [41] in pulmonary cells, we thought it important to describe a second control group with aqua only (the solvent of dexamethasone) to prove absent intrinsic effects of DMSO in our model (S+CP group).

Conclusion: Admixture of IKK-NBD peptide, but not dexamethasone, to surfactant improved gas exchange, lung function, and pulmonary edema by reducing chemokine generation and PMNL migration into the lung. Given as a single intratracheal dose, the effects of selective NF- κ B inhibition on surfactant “fortification” were obvious after approximately 12h and lasted for the whole observational period of 24h of mechanical ventilation. Contrary to the effect on smooth muscle relaxation by topical administration within several hours [42], dexamethasone failed to exert any significant clinical improvements in this neonatal animal model of respiratory failure. Our findings suggest that “fortification” of exogenous surfactant by selectively inhibiting NF- κ B might be a valuable therapeutic alternative to glucocorticoid treatment in the treatment of severe neonatal inflammatory lung disease.

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ACCEPTED MANUSCRIPT

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Figure captions**Figure 1**

PaO₂/FiO₂ over 24h of mechanical ventilation following airway lavage and surfactant administration with or without IKK-NBD peptide (S+IKK / S+CP), and with or without dexamethasone (S+Dexa / S+Aqua). C = control group receiving an air bolus only. Data are mean±SEM. Overall comparison of the 5 groups by repeated measures two-way (two-factorial) ANOVA for the observational period 1-12h: group differences p<0.001; time factor p<0.001, and for the period 12-24h: group differences p<0.001, time factor p=0.20. Comparison of S+IKK vs. S+Dexa: 1-12h group difference p=0.46, time factor p<0.001; 12-24h group difference p<0.05, time factor p=0.87.

Figure 2

Ventilation efficiency index (VEI) over 24h of mechanical ventilation. Overall comparison of the 5 groups by repeated measures two-way (two-factorial) ANOVA for the observational period 1-12h: group differences p<0.001; time factor p<0.001, and for the period 12-24h: group differences p<0.001, time factor p=0.22. Comparison of S+IKK vs. S+Dexa: 1-12h group difference p<0.01, time factor p<0.001; 12-24h group difference p<0.001, time factor p=0.19.

Figure 3

Total cell count in bronchoalveolar lavage fluid (BALF) of the intact lung and after 24h of mechanical ventilation. Data are mean±SEM. One-way ANOVA for comparison of all 5 groups, unpaired t-test for comparing S+IKK vs. S+Dexa.

Figure 4

Interleukin 8 (IL-8) and leukotriene B₄ (LTB₄) in broncho-alveolar lavage fluid (BALF) after 24h of mechanical ventilation. Data are mean±SEM. Overall comparisons of all 5 groups by one-way ANOVA, comparison of S+IKK vs. S+Dexa by unpaired t-test.

Figure 5

NF-κB activity in pulmonary cells by Western blots, separated into nucleus vs. cytosol, after 24h of mechanical ventilation. Data are mean±SEM. Overall comparisons of all 5 groups by one-way ANOVA, comparison of S+IKK vs. S+Dexa by unpaired t-test.

Table 1: Clinical parameters at baseline

	S+IKK (n=8)	S+CP (n=8)	S+Dexa (n=8)	S+Aqua n=8)	C (n=7)	p
weight (kg)	2.3±0.1	2.2±0.1	2.2±0.1	2.2±0.1	2.2±0.1	ns
lavages (n)	22±2	17±1	22±2	20±2	22±2	ns
saline lost in airways* (%)	10.7±1.1	12.3±1.1	9.0±0.3	10.6±1.3	9.5±0.7	ns
PaO ₂ /FiO ₂ (mmHg)	86±2	86±1	85±3	82±3	88±1	ns
PIP** (kPa)	2.45±0.16	2.43±0.23	2.47±0.06	2.51±0.10	2.55±0.10	ns

Treatment groups: surfactant+IKK-NBD peptide (S+IKK); surfactant+IKK-NBD control peptide (S+CP); surfactant+dexamethasone (S+Dexa); surfactant+aqua (S+Aqua); control (C). Mean±SEM. *Saline lost in airways” denotes the percentage of lavage fluid that was not recovered from the airways. **PIP = peak inspiratory pressure. ns = not significant.

Table 2: Lung function parameters and EVLW

		S+IKK (n=8)	S+CP (n=8)	S+Dexa (n=8)	S+Aqua (n=8)	C (n=7)	p treat (overall)	p S+IKK vs. S+Dexa
FRC (ml kg ⁻¹)	baseline	17.4±0.8	15.2±1.4	14.6±1.0	14.2±2.0	17.0±0.8	ns	
	1h	21.4±2.0	19.5±2.3	22.8±3.3	16.2±2.0	17.7±2.1		
	12h	32.9±3.6	22.9±2.0	25.8±3.4	22.8±4.7	18.4±2.0	<0.001/0.03	0.26/0.06
	24h	33.0±3.4	20.5±2.1	25.8±2.5	20.4±2.6	15.9±1.6	<0.001/0.48	0.01/0.72
C _{rs} (ml kPa ⁻¹ kg ⁻¹)	baseline	0.029±0.001	0.028±0.001	0.026±0.001	0.024±0.001	0.026±0.001	ns	
	1h	0.032±0.003	0.032±0.003	0.031±0.002	0.029±0.001	0.031±0.003		
	12h	0.062±0.005	0.037±0.004	0.041±0.002	0.040±0.004	0.036±0.002	<0.001/<0.001	<0.001/<0.001
	24h	0.056±0.006	0.037±0.004	0.041±0.003	0.036±0.004	0.033±0.004	<0.001/0.31	<0.001/0.28
V _A (ml kg ⁻¹)	baseline	2.7±0.2	2.4±0.4	2.4±0.2	2.8±0.3	2.5±0.3	ns	
	1h	1.9±0.3	2.0±0.3	1.8±0.2	1.8±0.3	2.4±0.3		
	12h	3.2±0.3	2.4±0.3	2.2±0.4	2.1±0.3	2.5±0.2	0.002/0.05	0.01/0.04
	24h	3.8±0.2	2.9±0.2	2.4±0.2	2.1±0.2	2.0±0.2	<0.001/0.51	<0.001/0.37
EVLW (ml kg ⁻¹)	baseline	52±3	48±5	47±4	54±5	51±3	ns	
	1h	39±2	40±3	37±3	39±3	40±1		
	12h	28±1	28±3	28±2	26±3	28±2	0.99/<0.001	0.76/0.004
	24h	24±1	31±2	29±2	27±4	34±3	0.41/0.76	0.26/0.79*

Treatment groups: surfactant+IKK-NBD peptide (S+IKK); surfactant+IKK-NBD control peptide (S+CP); surfactant+dexamethasone (S+Dexa); surfactant+aqua (S+Aqua); control (C). FRC = functional residual capacity; C_{rs} = dynamic compliance of the respiratory system; V_A = alveolar portion of tidal volume; EVLW = extra-vascular lung water. Mean±SEM. Comparisons of the 5 groups at baseline: not significant (ns). Comparisons of all 5 groups (treat, overall) and of S+IKK vs. S+Dexa by repeated measurement analysis, describing differences between hours 1-12 and 12-24 (treatment effect/time influence). * Unpaired t-test at 24h comparing S+IKK vs. S+Dexa: p<0.05.

Table 3: Blood leukocytes after 24h

	S+IKK (n=8)	S+CP (n=8)	S+Dexa (n=8)	S+Aqua (n=8)	C (n=7)	p treat (overall)	p S+IKK vs. S+Dexa
total cell count (cells μl^{-1})	7163±766	8086±676	7488±702	8443±1796	11207±1655	ns	ns
bands (%)	3.5±0.9	7.4±2.6	6.6±0.8	8.0±2.2	8.4±1.7	ns	0.03
PMNL (%)	55±4	54±4	59±4	56±3	53±5	ns	ns

Treatment groups: surfactant+IKK-NBD peptide (S+IKK); surfactant+IKK-NBD control peptide (S+CP); surfactant+dexamethasone (S+Dexa); surfactant+aqua (S+Aqua); control (C). Mean±SEM. Comparisons of all 5 groups (treat, overall) by one-way ANOVA; and of S+IKK vs. S+Dexa by unpaired t-test.

Figure 1

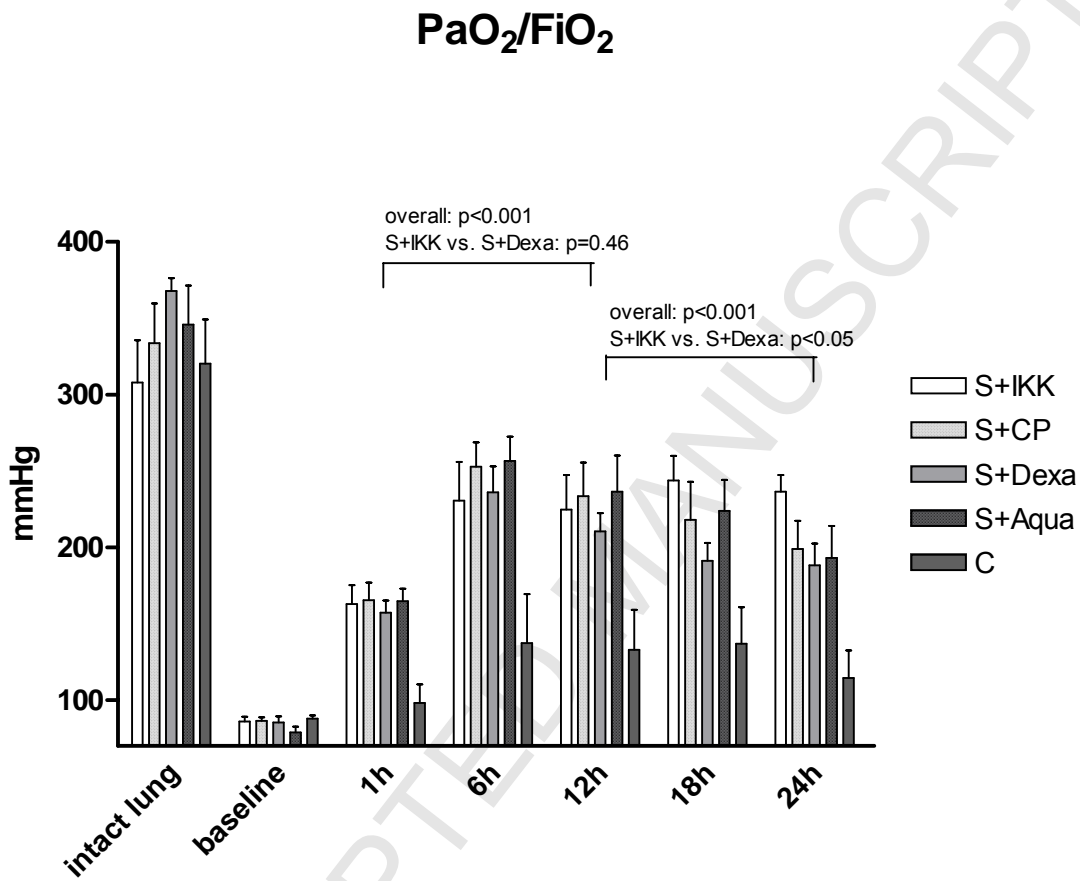


Figure 2

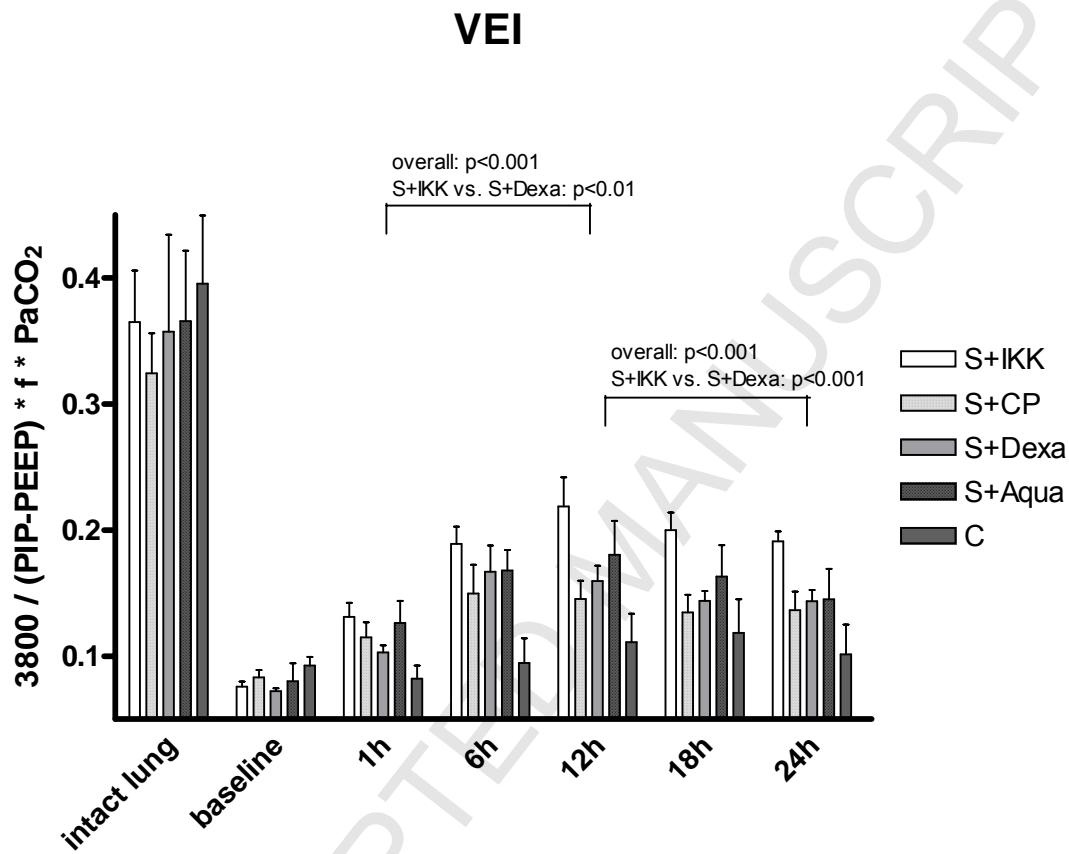


Figure 3

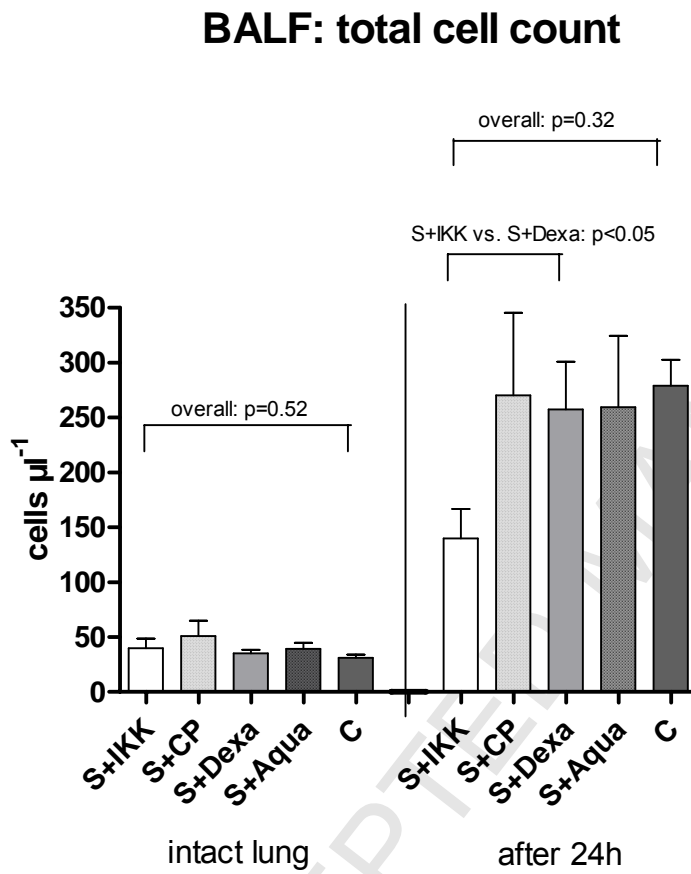


Figure 4

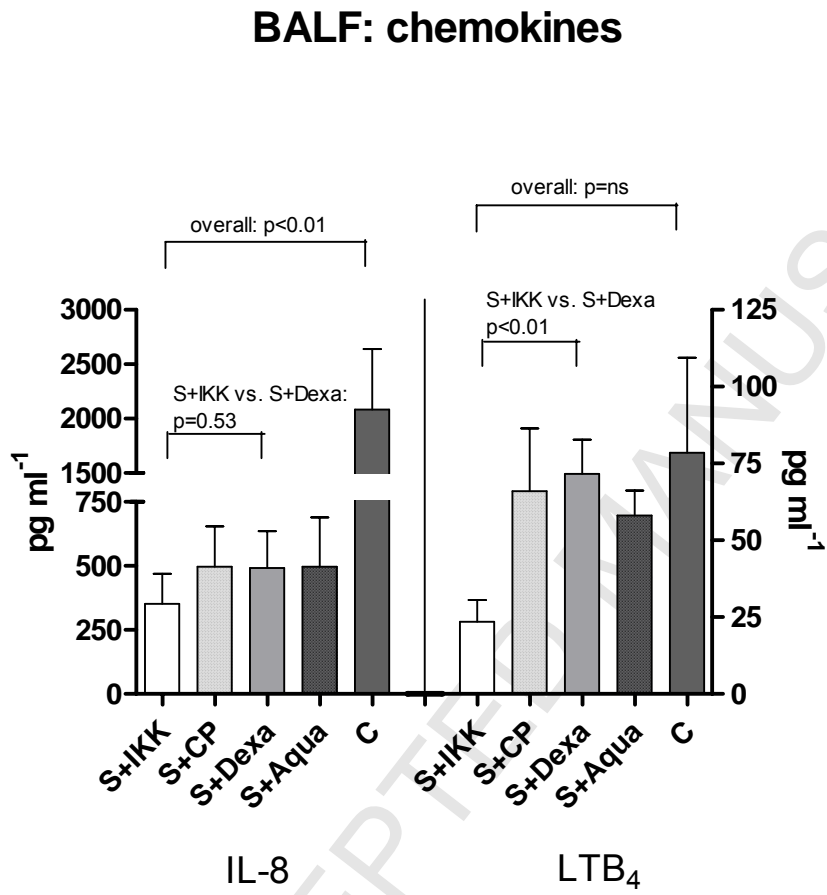


Figure 5

