Regulation of activity of nuclear factor-kB and activator protein-1 by nitric oxide, surfactant and glucocorticoids in alveolar macrophages from piglets with acute lung injury[1]

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ABSTRACT

AIM: To investigate whether acute lung injury (ALI) in ventilated piglets with bacterial infection affects NF-κB and AP-1 expression in alveolar macrophages (AM) and whether nitric oxide (NO), surfactant (Surf), glucocorticoids (GC) affect NF-κB and AP-1 activation in AM *in vivo* and *in vitro*. **METHODS:** The animals were intraperitoneally injected *Escherichia coli*, which caused ALI. Nuclear extracts of AM were analyzed by electrophoretic mobility shift assay (EMSA) for the nuclear factor-kappa B (NF-κB) and activation protein-1 (AP-1) expression. Detection of IκB-α protein was from cytoplasmic extract by Western blotting. Immunocytochemistry staining was used for intracellular location of p65 subunits of NF-κB. **RESULTS:** In *ex vivo* experiments, strikingly higher expression of NF-κB and AP-1 by EMSA was found 6 h after bacterial injection in contrast to the Normal group. In the NO, SNO, and GC groups, markedly attenuated NF-κB and AP-1 activation was observed. The NF-κB and AP-1 activation in Surf group showed lower levels of the expression. Immunoblotting of AM cytoplasmic extract showed low expression of IκB-α protein in the Control and Surf groups. The stronger expression was observed in the NO, GC, and SNO groups. AM of the Control and Surf groups showed intense nuclear staining, with decreased nuclear staining in the NO, GC and SNO groups. In *in vitro* experiment, it caused a significant increase in NF-κB and AP-1 activity in AM 1 h after exposure to lipopolysaccharides (LPS). In AM treated by LPS+SNP and LPS+GC, all showed decrease of DNA binding activity of NF-κB and AP-1 compared to those exposed to LPS+Surf. Immunoblotting of AM cytoplasmic extract showed that LPS stimulation of AM resulted in the low expression of IκB-α protein, which was not observed in the presence of SNP and methylprednisolone. However, the surfactant did not show such effect. LPS+Surf-exposed AM had intense nuclear staining, whereas decreased nuclear staining in the LPS+NO and LPS+GC-treated cultures was found, confirming a decrease in NF-κB activity. **CONCLUSION**: Activation of NF-κB was found in AM of ventilated piglets with bacterial ALI. NO and GS could prevent NF-κB and AP-1 activation *in vivo* and *in vitro*. Surfactant has limited effects on NF-kB and AP-1 activity.

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INTRODUCTION

Inflammatory reaction in bacterial infection-induced aute lung injury (ALI) is characterized by accumulation of large amount of macrophages and neutrophils in the lungs, causing increased production of immunally responsive cytokines and proteases as inflammatory mediators in the lungs[1], amplifying ventilator- and/or oxygen-associated acute and chronic damage. As a major effector of the innate immune system of the body, alveolar macrophages (AM) interact with other cells in airways and alveoli immediately after birth, resulting in generation of intracellular signaling cascades, leading to various effects or functions that make up the initial immune response in the lungs. Interation of AM and lipopolysaccharides (LPS) or bacterial infection causes a sequential activation of multiple signaling pathways and transcription factors in AM, resulting in gene transcription[2,3], orchestrating production of both pro- and anti-inflammatory mediators in the lungs. Two well-defined transcription factors influenced by inflammation are nuclear factor kappa B (NF-κB) and activation protein-1 (AP-1), which play a significant role in regulation of the production of many inflammatory mediators.

NF-κB is an ubiquitous transcription factor complex that directs transcription of many cytokines, adhesion molecules, and proinflammatory cytokine genes that promote corresponding mRNA translation and production of TNF-α, IL-1, IL-6, IL-8, IL-10, to name just a few of the genes with active NF-κB sites[4,5]. AP-1 belongs to a family of bZIP transcription factors, which is characterized by a basic domain required for sequence-specific DNA binding activity and a region containing heptad repeats of leucine and hydrophobic residues required for dimerization to other bZIP protein family members[6]. AP-1 transcription factors play critical roles in regulating target genes involved in adaptive responses to inflammation. It is speculated that activation of the inflammatory cascade involves the activation of both NF-κB and AP-1 in AM, which in turn induces and amplifies the expression of the cytokines such as TNF-α, IL-1, IL-6, IL-8, *etc*.

Acute inflammatory injury in ventilated infant lungs with bacterial infection, or other causes such as share force stretching and hyperoxia, may rely on activation of NF-κB and AP-1. There are limited evidence regarding the role of AM in these activation events[7], and fewer studies evaluated efficacy of NO[8], Surf and GC[9] on expression of NF-κB and AP-1 in AM from *in vivo* ALI

models with or without exposure to Gram negative bacterial toxin, or LPS. We hypothesized that activation of NF-κB may be central and pivotal to the development of pulmonary inflammation, and therefore investigated whether acute inflammatory injury in ventilated piglets with bacterial infection affects NF-κB and AP-1 activation in AM, and whether exogenous NO, Surf, and GC may alleviate expression of NF-κB and AP-1 activation in AM *ex vivo* and *in vitro*.

The lung exposed to risk factors such as meconium aspiration, hyperoxia, and infection frequently develops ALI. These injuries, occurring in developmentally and structurally immature lungs are thought to contribute to the pathogenesis of chronic lung disease (CLD). As a major effector of the innate immune system of the body, AM interact with other cells in airways and alveoli immediately after birth, resulting in generation of intracellular signaling cascades, leading to various effects or functions that make up the initial immune response in the lungs and cause activation of NF-κB and AP-1, resulting in gene transcription.

MATERIALS AND METHODS

***Ex vivo* experiments**

Animal model Healthy piglets were obtained from a local farm (Institute of Veterinary Medicine, Shanghai Academy of Agricultural Science, Shanghai). The animals, 3-5 weeks old and weighing 7-11 kg, were anesthetized and intubated. Each piglet was ventilated with a Servo 900C ventilator (Siemens-Elema, Solna, Sweden). Intravasular catheters were placed for blood gas measurement and fluid infusion as required for standard care. The animals were randomly allocated to 6 groups (*n*=6 per group). A Normal group was not subjected to bacterial injection but identical ventilation and management. The other animals were intraperitoneally injected with 5 mL of *Escherichia coli* (*E coli* ATCC 25922, 1×10^{12}-2×10^{12}/L), which caused ALI after 2-6 h. ALI was evidenced in animals with intraperitoneal injection of *Escherichia coli* by marked decrease of p_{aO_2}/F_{iO_2} less then 300 mmHg, and dynamic lung compliance (C_{dyn}) values more than 30 % compared with baseline. A control group received no additional treatment, and the other piglets were assigned to one of the four treatment groups: NO, inhaled NO at 10 ppm[10]; Surf, a porcine surfactant preparation[11] at phospholipids of 100 mg/kg body weight in 2.5 mL, instilled intratracheally; SNO, a combined treatment of surfactant and inhaled NO as for NO and Surf groups; GC, iv

methylprednisolone (Pharmacia & Upjohn, Puurs, Belgium) at 20 mg/kg. During the treatment, ventilator settings were regulated and standard care was given to maintain adequate gas exchange, blood pressure and electrolyte and fluid balance. The animal experiments were subjected to the other protocols also, hence the data regarding lung function *in vivo*, biochemistry and morphology are not reported herewith.

Airway aspiration procedure Three milliliters of normal saline was instilled in two 1.5-mL aliquots through a suction tubing introduced via a slide valve in the endotracheal tube connector until resistance was met. After the first aliquot administration the piglet was ventilated for a few minutes and a second aliquot was administered. Recovered fluid volume varied between 40 % and 80 % of the initial volume instilled, and two aliquots were combined in a sterile test tube and transported on ice to the laboratory for processing immediately. Airway aspitate (AA) samples for all time points were taken from the same animal: immediately after intratracheal intubation, 2 h after bacterial administration, establishment of ALI, and 6 h of the treatment, respectively.

AA cell counts One sample of the recovered AA fluid was used for microbiological and cytological study including total and differential cell count (DCC) after cytocentrifuge preparation. Manual DCC were performed for each AA specimen following Wright staining, and 300 cells per cytospin were counted.

Alveolar macrophage isolation AM in AA fluid were isolated by differential adherence. Obtained AA cells were collected by centrifugation at $400 \times g$ for 10 min at 4 ℃, then washed once with Hanks' balanced salt solution. RPMI-1640 medium (Glutamax, Gibco Life Technologies, Paisley, UK) was supplemented with 20 % fetal bovin serum (FBS) and 50 U penicillin G and 50 μg streptomycin per mL of medium. The washed cells were suspended at a concentration of $(3\text{-}5) \times 10^8$ cells/L in RPMI-1640 medium, added to 35-mm dishes, and incubated at 37 ℃ in 5 % CO_2 to allow adherence of AM. After 2 h of incubation, the nonadherent cells were discarded, and the monolayer, containing more than 95 % AM, as determined by Wright staining, was washed with PBS. AM obtained from bacteria-infected lungs were used for *ex vivo* experiments, and those from the Normal lungs, for *in vitro* experiments,.

Preparation of nuclear and cytoplasmic protein fractions Preparation of nucleus was extracted according to manufacturer's instruction of NE-PER™ nuclear and cytoplasmic extraction reagents kit (Pierce, Rockford, IL) and the extracts were kept at -80 ℃ for further use. The protein content of AM was determined with the bicinchoninic acid protein assay kit (BCA, Pierce).

Oligonucleotide labeling Oligonucleotides were labeled using a double-stranded consensus sequence NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3', Promega, Madison, WI) and AP-1 (5'-CGCTTGATGA-GTCAGCCGGAA-3', Promega) and $[\gamma\text{-}^{32}P]$-ATP (Yahui, Beijing, China). The reaction was catalyzed with T_4 polynucleotide kinase and incubated in 10×kinase buffer at 37 ℃ for 45 min. The reacion was stopped by heating at 65 ℃ for 10 min. Purification of the labeled oligonucleotides was performed with the QIAquick Nucleotide Removal kit (Qiagen, Valencia, Spain) according to the manufacturer's instructions.

Electrophoretic mobility shifting assay Nuclear extracts was analyzed by EMSA[10]. Binding reactions containing equal amounts of nuclear protein extract (10 μg) and 35 fmol of oligonucleotide were performed for 30 min in binding buffer (Promega). Reaction volumes were held constant to 15 μL. For experiments of specific competitor, a 50-fold excess of unlabeled ("cold") oligonucleotide was used and for experiments of nonspecific competitor, SP-1 (Promega, *O*-glycosylated transcription factor) were used. For a supershift assay, each sample was incubated with anti-p65 antibody 1 μg (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature before addition of the probe. To distinguish nonspecific binding of the AP-1, competition reactions were performed by adding 50-fold excess of nonradiolabeled AP-1 to the binding reaction mixture before electrophoresis. Reaction products were separated in a 4 % polyacrylamide gel in 0.5×TBE buffer (Tris base, boric acid and edetic acid) and analyzed by autoradiography. Autoradiograms were quantified using Imagequant analysis software (Shenteng, Shanghai, China).

Western blot for inhibitor of NF-κB (IκB-α) protein Cytoplasmic extract from cultured cells was prepared with the Pierce NE-PER™ kit (Pierce) and kept at -80 ℃. The protein content of AM was determined with BCA kit. Fifty micrograms of cytoplasmic protein extracts were separated by 12 % sodium dodecyl sulfate acrylamide gel electrophoresis, and electrotransferred (40 mA, 2 h) onto a nitrocellulose membrane (Bio-Rad, Hercules, CA), The membranes were blocked with 10 % FBS in PBS for 2 h. The membrane was

washed three times in 1×PBS-0.05 % Tween 20 and subsequently incubated with a rabbit polyclonal anti-IκB-α antibody (SC371, 0.5 mg/L; Santa Cruz, Santa Cruz, CA) for 1 h. The membrane was washed three times in 1×PBS-0.05 %-Tween 20 and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h. After successive washes, the membrane was developed with an enhanced chemiluminescence kit (SuperSignal Western Blotting kit, 34094, Pierce) according to manufacturer's instructions. Quantification of IκB-α levels was done with densitometer and Imagequant software (Shenteng, Shanghai, China).

Immunocytochemical staining for intracellular location of p65 AM were cultured on coverslips and the cells were fixed after 4 h, and stained with anti-p65 antibody. AM were plated (1×10^6 cells per well) in each well of 6-well plates containing glass coverslips, and allowed to adhere for 2 h, The coverslips were rinsed with PBS, and the adherent cells were fixed for 2 min in cold 4 % paraformaldehyde, and stored at -20 ℃ until stained. Before staining, the coverslips were rinsed in PBS (pH 7.4) for 10 min, and then blocked with 2 % goat serum for 15 min, briefly rinsed in PBS, and stained with anti-p65 antibody (Santa Cruz) for 60 min in a humid chamber. Following this, coverslips were washed thrice in PBS and biotinylated secondary antibody in 2 % goat serum was added, then the coverslips were incubated in a humid chamber for 1 h. The coverslips were incubated in peroxidase substrate for 5 min and then washed thrice in PBS, mounted on slides and sealed with clear nail polish.

In vitro experiments AM obtained from AA of normal piglets during early stage of mechanical ventilation were first centrifuged at 500×g at 4 ℃. The cell pellet was immediately maintained in RPMI-1640 medium with FBS and antibiotics and allowed to adhere for 2 h at 37 ℃ and 5 % CO$_2$ incubator. For *in vitro* experiments, AM were allowed to adhere and rest by indicated treatments. The best time of treatments and the best concentration of drugs were determined by pre-experiments. LPS (from *Escherichia coli*, Sigma, Lot 85H4092) was used at 1 μg/L 1 h for all experiments. Then the cells were either treated with sodium nitroprusside (SNP, NO donor) at 100 μg/L, or with Surf at 2 g/L, or with methylprednisolone at 0.3 μg/L. Nuclear extracts were then prepared after 2 h incubation as described in preparation of nuclear extracts and EMSA. Quantification of IκB-α was performed as above de-

scribed in preparation of cytoplasmic extracts and detection of IκB-α protein by the Western blotting. Immunocytochemical staining for intracellular location of p65 were also performed as described above.

Statistical analysis All the values are expressed as means±SD of at least five separate experiments in both *ex vivo* and *in vitro* experiments. Between-groups differences of the data from *ex vivo* and *in vitro* experiments were subjected to a one-way analysis of variance (ANOVA), and between-group differences were further compared with Student-Newman-Keuls *post hoc* test. Significance of the differences was defined as $P<0.05$.

RESULTS

Ex vivo experiments

NF-κB and AP-1 activation in AM from ventilated piglets No clear effect of enhanced expression of NF-κB and AP-1 activation was seen in all groups at early time of intratracheal intubation and 2 h after bacterial injection. However, strikingly higher expression of NF-κB and AP-1 by EMSA was found 6 h after bacterial injection (ALI was established) as reflected by substantially enhanced binding complexes of NF-κB and AP-1 from the nuclear extracts of AM in contrast to the same group 2 h after bacterial injection and the Normal group (without bacterial injection). In the NO and GC groups, markedly attenuated NF-κB and AP-1 activation was observed compared to the control group at 6 h. The NF-κB and AP-1 activation in Surf group showed moderately lower levels of the expression, but in the SNO group attenuated NF-κB and AP-1 activation remained obvious, compared to the control at 6 h after the treatment (Fig 1A-C).

IκB-α protein expression Immunoblotting of AM cytoplasmic extract with an anti-IκB-α antibody showed low expression of IκB-α protein in the Control and Surf groups. The stronger expression was observed in the NO, GC, and SNO groups (Fig 2A-B).

Immunocytochemical staining for intracellular location of p65 Minimal nuclear staining for p65 in the cytoplasm of AM in the Normal group was seen. AM of the control and Surf groups showed intense nuclear staining, with decreased nuclear staining in the NO, GC, and SNO groups, confirming a decrease in NF-κB activation. Despite the translocation of NF-κB to the nucleus, the cytoplasm remained with yellow light as a large portion of the NF-κB complex was retained
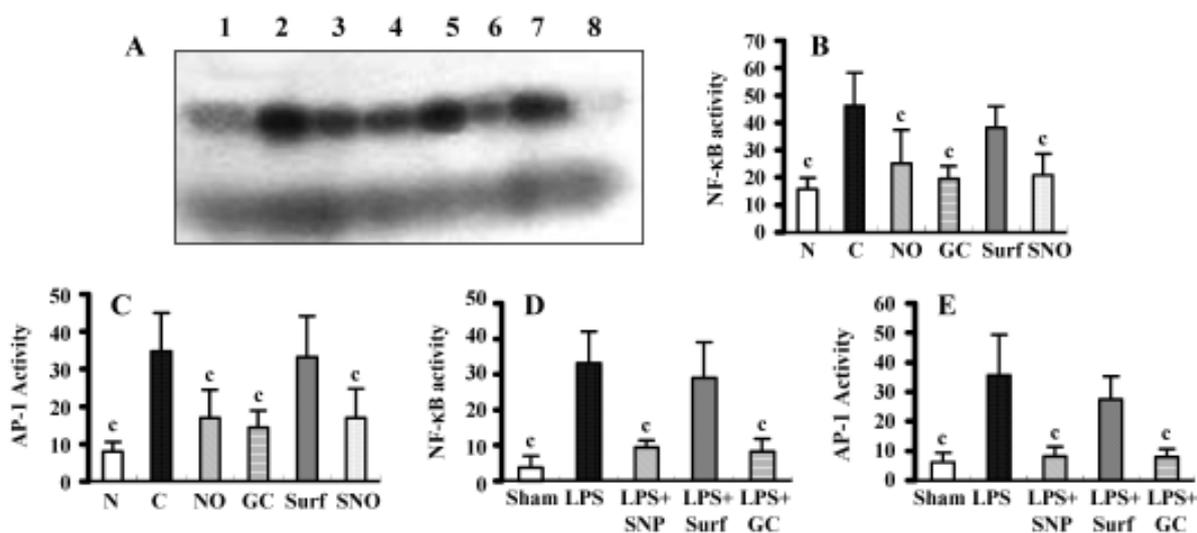
Fig 1. NF-κB and AP-1 activity in nuclear extracts of alveolar macrophages (AM). A: EMSA for NF-κB activity in nuclear extracts of AM *ex vivo*. Lane 1, normal (N); 2, control (C); 3, nitric oxide (NO); 4, glycocoticoids (GC); 5, surfactant (Surf); 6, Surf and NO (SNO); 7, nonspecific competitor; 8, specific competitor. B, C, D and E: Densitometric analysis for EMSA bands of nuclear extracts of AM by image analysis system. *n*=6. Mean±SD. B and C for NF-κB and AP-1 activity in AM *ex vivo*, respectively. $^cP<0.01$ *vs* Control; D and E for NF-κB and AP-1 activity in AM *in vitro*, respectively. $^cP<0.01$ *vs* LPS.
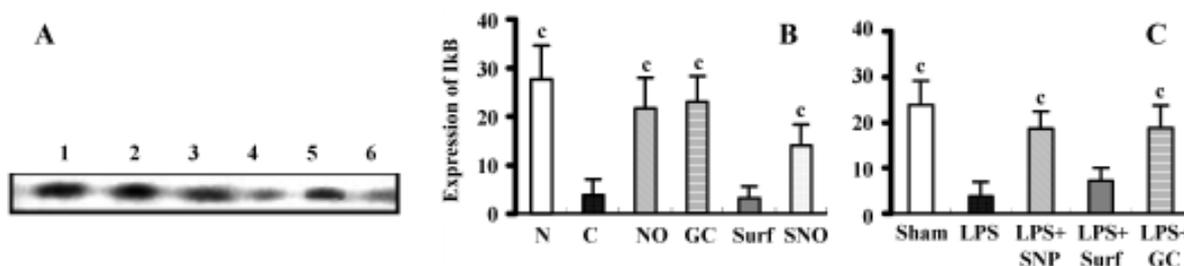

Fig 2. IκB-α levels in cytoplasmic extracts of AM. A: Western blotting for IκB-α levels in cytoplasmic extracts of AM. Lane 1, Normal (N); 2, GC; 3, NO; 4, Surf; 5, SNO; 6, Control (C). B and C: Densitometric analysis for Western blotting bands of cytoplasmic extracts of AM by image analysis system. *n*=6. Mean±SD. (B) for IκB-α levels in AM *ex vivo*. $^cP<0.01$ *vs* Control); (C) for IκB-α levels in AM *in vitro*. $^cP<0.01$ *vs* LPS.

in it (Fig 3).

In vitro experiment

NF-κB and AP-1 activation of LPS-stimulated AM One hour after exposure to LPS, it caused a significant increase in NF-κB and AP-1 activity in AM. In AM treated by LPS+SNP and LPS+GC, all showed decrease of DNA binding activity of NF-κB and AP-1. No increased NF-κB and AP-1 activity was observed in AM exposed to LPS+Surf compared to those exposed to LPS only (Fig 1D-E).

Immunoblotting of IκB-α protein Immunoblotting of AM cytoplasmic extract with the anti-IκB-α antibody showed that LPS stimulation of AM resulted in the low expression of IκB-α protein, which was not observed in the presence of SNP and methylprednisolone. However, the surfactant did not show such ef-

fect (Fig 2C).

Immunocytochemical staining for intracellular location of p65 LPS+Surf-exposed AM had intense nuclear staining, whereas decreased nuclear staining in the LPS+NO and LPS+GC-treated cultures was found, confirming a decrease in NF-κB activation.

DISCUSSION

AM is an important source of cytokines and growth factors in the lungs[5], and many cytokines are regulated by the redox-sensitive transcription factor, NF-κB[4]. NF-κB consists of a heterodimer of p50 and p65 subunits, which are retained in most cells in the cytoplasm in complex with inhibitory proteins of the inhibitor-κB family. In unstimulated cells, NF-κB resides in
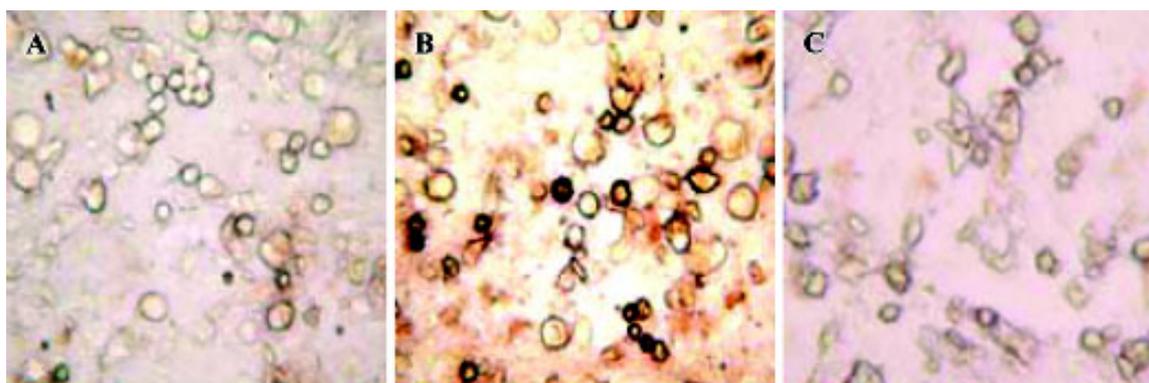
Fig 3. Immunocytochemical staining for intracellular location of p65. A: normal group, AM were not activated and the p65 (NF-κB) staining remained localized to the cytoplasm. B: control group, AM were activated and the NF-κB migrated to the nucleus and nuclei appear yellow. C: NO group, nuclear staining with p65 was decreased in AM compared with the control group.

the cytoplasm as a dimer of protein components known as Rel family member (eg, p50, p65[12], which is bound to an IκB. Upon stimulation or activation, IκB is phosphorylated and released from the complex, after which the complex undergoes proteolytic degradation and the Rel proteins migrate to the nucleus and bind to the cognate sites in the promoter regions of the genes for many inflammatory cytokine and growth factors[5], resulting in their transcription[12]. Increasing evidence suggests an important role for NF-κB in the pathogenesis of lung inflammation. Results from the study have shown that NF-κB regulates gene expression of cytokines[4]. These *in vitro* findings are supported by studies in humans with ARDS that enhanced NF-κB activation was found in AM recovered by AA[13]. Some recent *in vivo* studies have demonstrated that lung NF-κB activation is suppressed by NO[8] and GC[9], resulting in decreased proinflammatory mediator expression and reduced inflammatory injury[14]. Thus, it appears that activation of NF-κB may be central and pivotal to the development of pulmonary inflammation.

The transcription factor AP-1 is a known stress response complex that functions in signal transduction by inducing the expression of specific downstream genes[15]. The AP-1 complex consists of various heterodimers of fos/jun proteins or homodimers of jun/jun proteins. Several mechanisms are believed to be involved in regulating AP-1 transcriptional activity, including differential gene expression of fos or jun, conformational alterations, posttranslation modification, and altered DNA- binding specificities of the heterodimers. AP-1 DNA-binding activity can be modulated by a direct association between thioredoxin and Ref-1[16].

There is little direct evidence regarding the role of AM in these activation events. We observed that AM from piglets had enhanced AP-1 binding activity after bacterial exposure *in vivo* and LPS exposure *in vitro*. The underlying mechanism of this alteration requires further investigation to correlate it with severity of the inflammation and injury.

We also investigated whether NO, Surf and GC affected NF-κB and AP-1 activation *in vitro*. In AM from the Normal animal lungs and stimulated by LPS subsequently *in vitro*, we compared their response in the absence and presence of NO, Surf, and GC (methylprednisolone), and found that NO and GC suppressed NF-κB and AP-1 activity in similar magnitude, but the Surf had apparently less effect on it. NO is an inorganic free radical and a remarkably versatile biological messenger. The chemical properties of NO are crucial in defining its biological roles as both a transcellular signal in the cardiovascular and nervous systems and a cytotoxic antipathogenic agent released during inflammatory response. NO is an important endogenous regulatory molecule synthesized in various cells of the lungs, and involved in many diverse physiologic processes[17,18]. However, there is scarce information about NO metabolite affecting the activating process of NF-κB and AP-1. It has been shown that NO donor agents suppress NF-κB activation in human endothelial cells *in vitro* by increasing the level of IκB[19]. In contrast, studies with human peripheral blood lymphocytes have demonstrated activation of NF-κB in the presence of NO donors[20]. Currently, there is no explanation for these conflicting results. Subtle differences in experimental design, cell type specialty, difference of NO donors,

and prestimulation of cells may account for the different results. Our *ex vivo* findings indicate that NO is anti-inflammatory through decreasing NF-κB and AP-1 activation and therefore has a potential role in protection of the lungs from ALI.

Understanding NO function may lead to ways of modulating inflammatory response. SNP is often used as an NO donor for clinical and laboratory experiment. The vasodilator effects of SNP are believed to be largely mediated by NO, but its mechanism of action remains uncertain[21]. In the present study, we found that NO decreased NF-κB and AP-1 in AM *in vitro*. IκB-α protein levels were greater in cytoplasmic extraction from LPS+SNP-treated than in those from LPS-treated cells. These results indicate that NO prevented NF-κB activation by maintaining a steady-state level of IκB-α protein, an effect that may be mediated by increase of IκB synthesis and/or decrease of IκB degradation.

Despite wide use of GC in clinical disorders, the mechanism of anti-inflammatory action of GC is not completely defined. GC effects are mediated through an intracellular receptor, the GC receptor, a member of the steroid hormone receptor superfamily[22]. Upon hormone binding, the cytoplasmic GC receptor can enter into nucleus, dimerize, and bind to specific DNA sequences located in the 5' promoter region of many genes, leading to modulation of gene transcription[23]. The activated GC receptor may also interact with other transcription factors such as NF-κB and AP-1, to regulate gene transcription[24]. *In vitro* studies have shown that some of the anti-inflammatory actions of GC are due to inhibition of NF-κB[25,26]. Although GC are useful in the treatment of some inflammatory diseases, their role for mitigation of severity of pulmonary inflammatory injury in mechanical ventilation with bacterial infection remains uncertain. Because previous studies with GC in animal models of lung injury have shown variable results[27], our results indicate that the actions of GC on inflammatory process are complex and certainly involve NF-κB and AP-1.

Surfactant treatment is a standard care for premature newborn infants with respiratory distress syndrome due to surfactant deficiency and lung immaturity. Surfactant suppresses streptococcal proliferation in neonatal pneumonia, and also has a potential to inhibit proinflammatory cytokine release from AM or mononuclear leukocytes[28]. The recent study has shown that SP-A dramatically inhibits activation of NF-κB in AM stimulated with LPS[28]. Another study showed that phospholipids of surfactant can downregulate the immune response[29]. In the *in vitro* experiments, release of inflammatory cytokines by human AM was inhibited by lipid components of the surfactant[30]. Thus, the surfactant lipids may have anti-inflammatory actions in the alveolar space. Nevertheless, the exact mechanisms are incompletely understood. We found that in the *ex vivo* study, surfactant (phospholipids with SP-B and SP-C, no SP-A) had variable effects on NF-κB and AP-1 activation in piglet AM, but it had no effects in inhibition of activation of NF-κB and AP-1 in AM stimulated with LPS *in vitro*. Our results suggest that the anti-inflammatory effects of the surfactant lipids may not be exerted through NF-κB and AP-1 signaling pathways, and is consistent with another author[31].

AM activation is an initial event in the genesis of lung inflammatory reactions. Furthermore, NF-κB activation in AM *in vivo* occurs prior to NF-κB activation in whole-lung tissues, suggesting that products of activated AM are required to stimulate nuclear translocation of NF-κB in other lung-cell types[32]. The present data support that NF-κB activation is pivotal in the pathogenesis of lung inflammation. In our *in vivo* experiments by examining freshly isolated AM from normal piglets and ventilated piglets with bacterial infection, we have established a method to obtain *in vivo* "silent" or inflammation activated AM as characterized by their response to *in vitro* stimulations leading to up- or downregulation of expression of NF-κB intracellularly. This enables further study in the mechanism of interaction between inflammatory cells and lung cells in ALI, and growth factor-mediated lung cell proliferation and reparation.

We conclude that interaction of LPS or bacterial infection with AM causes a sequential activation of NF-κB and AP-1 signaling pathways, resulting in gene transcription. Activation of NF-κB and AP-1 in AM of ventilated piglets with bacterial infection may be downregulated by NO and GC. Surfactant has limited effects in regulation of NF-κB and AP-1 activity. Further studies are required to elucidate the role of NF-κB in mediating signal transduction in pulmonary cell proliferation and reparation in septic ALI, and its modulation in preventing biotrauma as seen in ARDS.

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