Surfactant Blocks Lipopolysaccharide Signaling by Inhibiting both Mitogen-Activated Protein and IκB Kinases in Human Alveolar Macrophages

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Surfactant plays an important role in lung homeostasis and is also involved in maintaining innate immunity within the lung. Lipopolysaccharide (LPS) from gram-negative bacteria is known to elicit acute proinflammatory responses in lung diseases such as acute respiratory distress syndrome and pneumonia, among others. Our previous studies demonstrated that the clinically used, natural surfactant product Survanta inhibited proinflammatory cytokine secretion from LPS-stimulated human alveolar macrophages. Here we investigated the effect of Survanta on mitogen-activated protein (MAP) and IκB kinases. Survanta blocked LPS-induced activation of nuclear factor-κB, a key regulatory transcription factor involved in cytokine production, by preventing phosphorylation of IκBα, and its subsequent degradation. IκB is phosphorylated by specific kinases (IKK) before degradation. Survanta inhibited activity of both α and β subunits of IKK, thereby delaying the phosphorylation of IκB. Interestingly, IKK-α is predominant in alveolar macrophages, whereas IKK-β predominates in monocytes. Survanta also inhibited extracellular signal–regulated kinase and p38 MAP kinase activity induced by LPS. Data are the first to show that surfactant may regulate lung homeostasis in part by inhibiting proinflammatory cytokine production through reduction of IKK and MAP kinase activity.

Surfactant is a complex mixture of proteins and lipids synthesized by alveolar type II cells and secreted into the alveolar lining fluid. The biophysical properties of surfactant are responsible for stabilization of the alveoli, prevention of microatelectasis, and facilitation of gas exchange (1, 2). Previous studies have demonstrated that surfactant attenuates cytokine secretion from alveolar macrophages (3, 4), suggesting that surfactant may serve an endogenous immunoregulatory role in the normal alveolar space. This may be of significance, because surfactant function is disrupted in many lung diseases, including acute respiratory distress syndrome, idiopathic fibrosis, and asthma (1). Excess cytokine and chemokine production have been implicated as contributors to the pathology of all of these diseases (5–8).

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Abbreviations: enzyme-linked immunosorbent assay, ELISA; lipopolysaccharide, LPS; mitogen-activated protein, MAP; MAP kinase, MAPK; macrophage inflammatory protein-1α, MIP-1; nuclear factor-κB, NF-κB; tumor necrosis factor, TNF; whole cell extract, WCE.

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A major component of innate immunity in the lung is the alveolar macrophage, which responds to microbial challenge by releasing an array of cytokines and chemokines that mediate inflammation. In previous *ex vivo* studies of from healthy control human alveolar macrophages challenged with endotoxin or *Staphylococcus aureus*, we observed that surfactant dose-dependently reduced gene expression and protein secretion of inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (4). Crucial to the activation of many cytokine genes is the transcription factor nuclear factor (NF)-κB. We have also demonstrated that surfactant inhibits NF-κB activation in the monocytic cell line THP-1 (9).

The MAP kinases (MAPKs) are a family of enzymes that link cell surface receptors to regulatory targets that include nuclear as well as cytoplasmic proteins (reviewed in Ref. 10). In blood monocytes and macrophages, lipopolysaccharide (LPS) induces various members of the MAPK family, including p38 and extracellular signal–regulated kinase (ERK). Carter and coworkers have shown that both p38 and ERK are critical for LPS-induced cytokine release in monocytes and alveolar macrophages (11).

Macrophage inflammatory protein-1α (MIP-1) is produced abundantly by alveolar macrophages and is a member of the CC family of chemokines, which attract monocytes, dendritic cells, eosinophils, and lymphocytes to sites of injury (12). Similar to many inflammatory cytokines and chemokines, MIP-1 is regulated at least in part by NF-κB (12). High levels of MIP-1 are found in bronchoalveolar lavage fluids in many acute and chronic lung diseases (12). MIP-1 null mice have reduced inflammation in response to viral infections (13) and MIP-1 antibodies abrogate eosinophil influx in animal models of allergic inflammation (reviewed in Ref. 14). Taken together, these observations suggest a critical role for MIP-1 in the pathology of numerous lung diseases. The effect of surfactant on MIP-1 secretion by human alveolar macrophages has not been previously investigated.

The present study was undertaken to determine the mechanism of signaling disruption by surfactant. We hypothesized that surfactant would inhibit cytokine secretion by disrupting both IKK and MAPK signaling in human alveolar macrophages. The results demonstrate that surfactant targets multiple check points in cell signaling pathways.

Materials and Methods

Reagents

S*almonella typhimurium* LPS was obtained from Sigma (St. Louis, MO) and used at 0.5 μg/ml for all experiments. Survanta was a

gift from Ross Laboratories (Columbus, OH) and used at the previously determined optimum concentration of 1,000 μg/ml (4). Survanta is a natural bovine lung extract containing phospholipids, neutral lipids, fatty acids, and surfactant-associated proteins B and C, to which DPPC, palmitic acid, and tripalmitin are added to standardize the composition.

Alveolar Macrophages

Fiberoptic bronchoscopy with bronchoalveolar lavage was performed as previously described (15). The study population consisted of healthy volunteers, 18–65 yr of age, with no lung disease and on no medication. All volunteers provided written informed consent and the study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. Alveolar macrophages were obtained by adhering cells from bronchoalveolar lavage as previously described (15, 16). Nonadherent cells were removed by washing. The adherent cell population consisted of $> 99\%$ macrophages. Alveolar macrophages were cultured overnight before *in vitro* treatment. For each experiment, adhered cells were treated with LPS ± Survanta or left untreated (US).

Preparation of Whole Cell Extracts

After overnight incubation, macrophages were treated with LPS ± Survanta or left untreated for 4 h. Survanta did not adversely affect cell viability as measured by trypan blue dye exclusion and cell adherence. Cells were harvested and whole cell extracts (WCE) prepared as previously described (15). The protein content of WCE was measured by bicinchoninic acid protein assay method (Pierce, Rockford, IL).

Western Blot Analysis

Cells were washed once with ice-cold phosphate-buffered saline and lysed as described (15). Protein concentrations were measured and 10 μg of the cell lysate was mixed with 1:1 sample buffer, boiled and analyzed on a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Immobilon-P membranes. After blocking membranes, primary antibody to IκBα, p38, or ERK (Santa Cruz Biotechnology, Santa Cruz, CA) or phosphorylated IκBα, ERK, or p38 (Cell Signaling, Beverly, MA) was applied at 1:1,000 dilution for 1 h at room temperature. After secondary antibody application and washing, bands were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Construction of GST-IκBα

This plasmid was constructed as previously described (17). Briefly, amino acids 1–54 were cloned into the Nco I and Xho I site of pGexKG.

Immunoprecipitation and Kinase Assay

Proteins were immunoprecipitated from WCE after preclearance by adding antibody (2 μg) and 50 μl of A/G Sepharose. After rotation for overnight at 4°C, immunoprecipitates were washed with lysis buffer three times and then with kinase buffer (20 mM HEPES-KOH pH7.4, 25 mM β-glycerophosphate, 20 mM $MgCl_2$) twice. The kinase assay was performed in a final volume of 20 μl of kinase buffer containing 2 μg of bacterially purified GST-IκB α, 20 μM of ATP, and 5 mCi[P^{32}] ATP. After incubation for 20 min at 30°C, the reaction was stopped by addition of 2× sample buffer. After separation by SDS-PAGE, the gel was dried and autoradiographed.

Preparation of RNA and Analysis

Total RNA was prepared from adhered macrophages by RNAeasy protocol (Qiagen, Valencia, CA). Gene expression was quantified by real time RT-PCR using the ABI prism 7,000 detection system (Taqman; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. To minimize the error of cross contamination, every sample was done in duplicate using primer/probe sets for a housekeeping gene (GAPDH) and MIP-1α (ABI). Threshold cycle (CT) values for genes of interest were normalized to GAPDH and used to calculate the relative quantity of mRNA. Data are expressed as fold change relative to control values.

Analysis of Chemokines

MIP-1α was analyzed in duplicate samples of cell-free supernatant fluids from 24-h macrophage cultures by enzyme-linked immunosorbent assay (ELISA; Endogen, Cambridge, MA). Assay sensitivity ranged from 25–1,000 pg/ml, and the coefficient of variation was $< 10\%$.

Statistical Analysis

Data were analyzed by one-way ANOVA and Student's *t* test using Prism software (GraphPad, Inc., San Diego, CA). Significance was defined as $P \leqslant 0.05$. Means ± SEM are provided.

Results

MIP-1 Secretion Is Downregulated by Survanta

The effect of Survanta on LPS-stimulated alveolar macrophage MIP-1 production after 24 h was evaluated by ELISA (Figure 1). As expected, treatment with LPS alone increased MIP-1α secretion. Simultaneous treatment with Survanta inhibited MIP-1α secretion from alveolar macrophages ($P = 0.01$). To determine whether this inhibitory effect was stimulus specific, granulocyte macrophage colony-stimulating factor (GM-CSF), as an endogenous stimulator, was used. GM-CSF stimulation significantly elevated MIP-1α secretion ($P =$ 0. 01). Survanta also decreased GM-CSF–induced MIP-1 α secretion ($P = 0.04$) (Figure 1).

Chemokine Gene Expression Is Downregulated by Survanta

Alveolar macrophage gene expression was quantified by real-time RT-PCR analysis. Alveolar macrophages were isolated and adhered overnight. Cells were then treated with LPS ± Survanta for 24 h and total RNA was isolated. Treatment with LPS alone increased alveolar macrophage gene expression of MIP-1α, when compared with unstimulated cells. Simultaneous treatment with LPS and Survanta reduced the MIP-1α gene expression ($P = 0.01$; Figure 2). Treatment with GM-CSF also increased MIP-1α gene expression, although to a lesser extent, when compared with LPS-induced MIP-1α gene expression (data not shown).

Survanta Delays IκB Degradation

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degra-

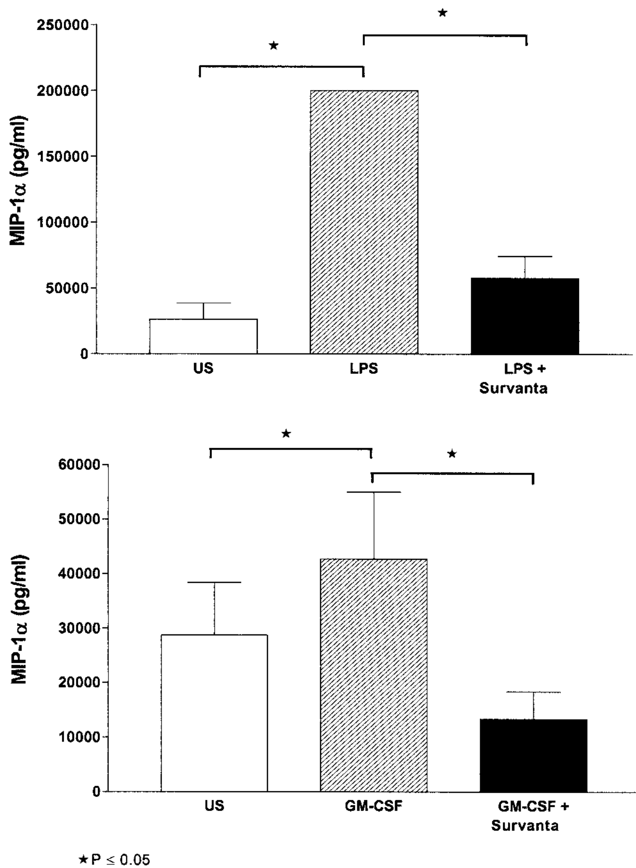

Figure 1. (*Upper panel*) Survanta inhibits LPS induced MIP-1α secretion from alveolar macrophages. Human alveolar macrophages from three different individuals were incubated with LPS (0.5 μg/ml ± Survanta 1 mg/ml) for 24 h or left untreated. Supernatants were collected and cytokines measured by ELISA ($P = 0.01$).(*Lower panel*) Survanta inhibits GM-CSF–induced MIP-1α secretion from alveolar macrophages: Human alveolar macrophages from three different individuals were incubated with (GM-CSF [1,000 U/ml] ± Survanta [1 mg/ml]) for 24 h or left untreated. Supernatants were collected and cytokines measured by ELISA ($P = 0.03$).

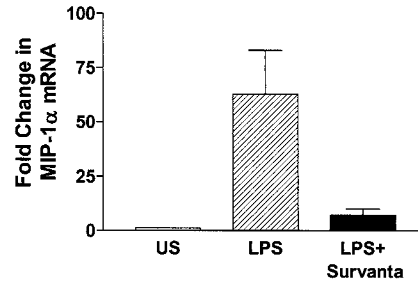

Figure 2. Survanta inhibits LPS-induced MIP-1α gene expression. Total RNA was prepared from three different individuals and subject to real time PCR analysis by Taqman. Relative quantity of RNA expression was measured and expressed as fold change ($P = 0.01$)

dation of IκB-α (18, 19). To determine whether Survanta inhibited NF-κB activation by blocking IκB phosphorylation and degradation, immunoblot analysis of cell lysate from unstimulated cells or from LPS ± Survanta–treated cells was performed. Figure 3 shows that LPS enhanced IκBα loss (*lanes 2–4*) in a time-dependent manner. Addition of Survanta delayed the disappearance of IκBα (compare *lanes 2* and *5* with *lanes 3* and *6*). To ascertain that Survanta effects on IκB were through phosphorylation, the same blot was immunoprobed using an antibody specific for phosphorylated IκBα (Figure 3). LPS induces phosphorylation of IκBα (*lanes 2–4*). LPS induced significant phosphorylation within 45 min and continued until 75 min. Simultaneous addition of Survanta inhibited phosphorylation of the IκB protein (compare *lane 3* with *lane 6*). These results suggest that Survanta inhibits phosphorylation of IκB and its subsequent degradation.

Survanta Inhibits both IKKα and β-Kinase Activity

IκBα is phosphorylated by IKK complexes (17). LPS activates IKKβ in human monocytes, THP-1 cells, and the mouse macrophage cell RAW 264.7 (20). IKK activity has not been studied in human alveolar macrophages. Because Survanta delayed the phosphorylation of IκB, we investigated the effect of Survanta on LPS-induced IKK activity. The kinase complex was immunoprecipitated from unstimulated and LPS ± Survanta–treated cells. Kinase activity was measured *in vitro* using GST-IκBα as substrate. Figures 4A and 4B show that both IKKα and IKKβ were activated by LPS. Unstimulated cells had no basal kinase activity. Addition of Survanta inhibited both IKKα and IKKβ kinase activity. Surprisingly, LPS-stimulated IKKα activity in alveolar macrophages is greater than the activity of IKKβ (Figure 4). In contrast, studies with the monocytic cell line THP-1 showed that IKKβ was dominant (21).

Survanta Inhibits ERK and p38 Kinase in Human Alveolar Macrophages

Figures 5A and 5B show the time-dependent activation of ERK and p38 kinase and the effect of Survanta on LPS-induced MAPK activation. Phosphorylation of ERK and p38 is almost undetectable in unstimulated condition (*lane 1* at *left* in Figures 5A and 5B). LPS treatment increases both ERK and p38 phosphorylation rapidly in a time-dependent manner (*2nd* and *4th lane* from *left*). In the presence of Survanta, both the phosphorylation of p38 and ERK is decreased (*3rd* and *5th lanes* from *left*).

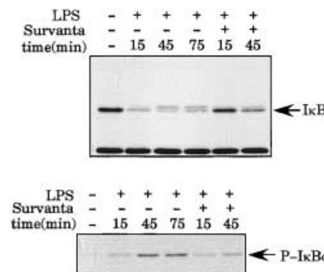

Figure 3. Survanta delays LPS-induced IκBα phosphorylation. WCE were prepared from unstimulated (*lane 1*), LPS-treated (*lanes 3*, *4*, and *5*), or LPS + Survanta–treated (*lanes 6* and *7*) cells for the times indicated. *Upper panel* shows the phosphorylation of IκBα. The blots were stripped and reprobed for phosphorylated IκBα (*lower panel*). Constitutive band shown indicates equal loading. Results are representative of three separate experiments.

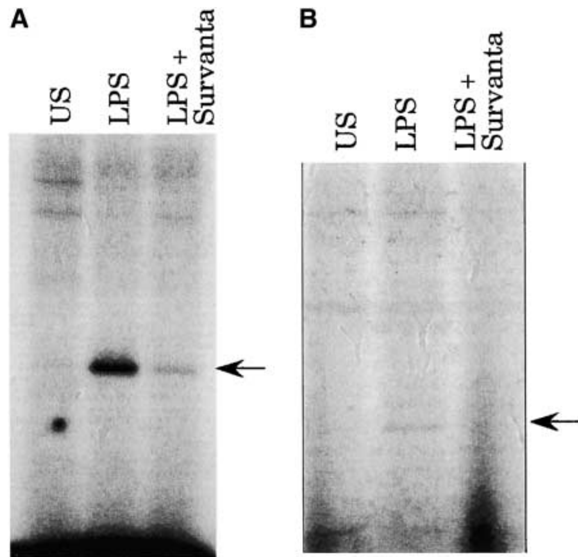

Figure 4. Survanta inhibits IKK activity. Cells were unstimulated (US, *lane 1*), treated with LPS (*lane 2*), or treated with LPS + Survanta (*lane 3*) for 20 min. IKK kinase activity was determined in the WCE after immunoprecipitating with IKKα (*A*) and IKK β (*B*) antibodies using GST-IκBα (1–54) peptide substrates.

Discussion

We have shown that Survanta inhibits LPS-stimulated MIP-1α secretion by alveolar macrophages and that this effect is not stimulus-specific because GM-CSF–stimulated MIP-1 was also inhibited**.** Furthermore, MAPK activation in human alveolar macrophages was reduced by Survanta. Finally, we have shown for the first time that Survanta inhibits both IKKα and β activity, thus delaying phosphorylation of IκB. Taken together, these observations suggest that surfactant is a potent downregulator of chemokine production and inhibits both NF-κB and MAPK pathways. In many airway disorders, inflammation plays a central role and is associated with morbidity and mortality (5–8). Surfactant modulates several inflammatory processes, including cell proliferation and the release of inflammatory mediators (3, 4, 22, 23). Surfactant deficiencies may contribute to respiratory failure (1, 2). Previous studies by several groups including ours have shown that synthetic surfactant and several natural surfactant preparations downregulate inflammatory cytokine production by both monocytes and alveolar macrophages (4, 9). NF-κB regulates both innate and adaptive responses and is activated by wide range of stimuli including pathogens, stress signals, and inflammatory cytokines (24). We previously demonstrated that a synthetic surfactant (Exosurf) blocked NF-κB activation in THP-1 cells (9). Exosurf is no longer used clinically because the two commercially available naturally derived surfactant preparations, Survanta and Infasurf, have greater efficacy in the treatment of neonatal respiratory distress syndrome (25).

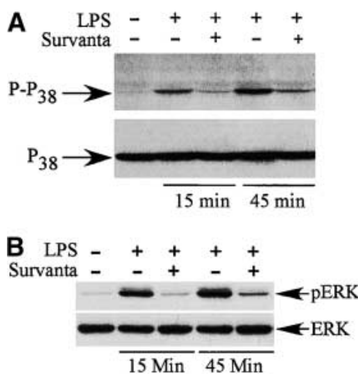

Figure 5. Survanta inhibits ERK and p38 phosphorylation. WCE were prepared from unstimulated (from the left *lane 1*), LPS-treated (*lanes 2* and *4*) or LPS + Survanta–treated (*lanes 3* and *5*) cells for the times indicated. *Top panel* in *A* shows the phosphorylated p38 and the *bottom panel* p38 only (the blots were stripped and reprobed for p38). Constitutive band shown indicates equal loading. *Top panel* in *B* shows Erk phosphorylation (from left: *lane 1*, US; *lanes 2* and *4*, LPS-treated; *lanes 3* and *5*, LPS + Survanta–treated) and the *bottom panel* shows ERK. Results are representative of three separate experiments.

Molecular regulation of inflammatory cytokines is a complex process, and the transcription factor NF-κB plays a pivotal role. Sequential phosphorylation, ubiquitination, and degradation of the inhibitory component IκB permits NF-κB/Rel protein to move to the nucleus (18, 19, 26). A high molecular mass kinase complex has been described to contain kinase activity specifically for Ser 32 and Ser 36 of IκB-α (17, 24). In the present study, we have demonstrated for the first time that surfactant inhibits IκB phosphorylation and degradation by blocking both IKKα and IKKβ kinase activity. Furthermore, in contrast to previous studies with the monocytic cell line THP-1 (21), the dominant IKK in human alveolar macrophages appears to be α, although β, which is much less active, is also reduced by surfactant.

Carter and colleagues (11) have shown that both the ERK and p38 kinase pathways are activated in alveolar macrophages stimulated by LPS and are necessary for optimal cytokine gene transcription. Survanta inhibited ERK and p38 activation. These results suggest that Survanta in part regulates cytokine production in alveolar macrophages by interfering with MAPK signaling.

A recent report has suggested that surfactant associated protein C (SP-C) or a synthetic analog of SP-C binds LPS and prevents LPS binding to mouse macrophages and thus TNF secretion (27). However, these effects were observed at LPS concentrations of 40 ng/ml. At concentrations higher than 50 ng/ml the ability of synthetic SP-C to neutralize the LPS decreased progressively (27). In our system we used 500 ng/ml of LPS. We addressed the possibility of LPS-surfactant binding in earlier experiments and found no evidence for binding. We found that cells could be pretreated with LPS for 1 h and then washed and treated with a synthetic surfactant (Exosurf) and TNF secretion was still blocked (4). Finally, the effects we observed with both Exosurf and Survanta do not appear to be stimulus-specific because *Staphylococcus aureus*–, interleukin-1– (previous studies) (4), and GM-CSF (present study)–stimulated cytokine secretion were also blocked. Taken together, these observations suggest that the inhibitory effects of surfactant we observed with human alveolar macrophages are not due to surfactant binding to LPS.

In summary, our findings suggest pulmonary surfactant plays a crucial role in maintaining lung homeostasis by serving as an endogenous downregulator of cytokine pro-

duction by human alveolar macrophages. Surfactant blocks both chemokine and inflammatory cytokine production by inhibiting IKK activity, which prevents the degradation of IκB and subsequent NF-κB activation. Furthermore, MAPK signaling was blocked by surfactant. These observations suggest that surfactant, in addition to mechanical benefits to lung function, blocks cellular responses to exogenous stimuli at multiple checkpoints and therefore may be useful in reducing pulmonary inflammatory responses.

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