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Molecular mechanisms of lipopolysaccharide-caused induction of *surfactant protein-A* gene expression in human alveolar epithelial A549 cells

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ABSTRACT

Surfactant proteins (SPs) participate in the physiological and pathophysiological regulation of sepsis-induced acute lung injury. Lipopolysaccharide (LPS), a Gram-negative bacterial outer membrane component, is one of the major causes of septic shock. This study was designed to evaluate the effects of LPS on the regulation of *SP-A* and *SP-D* gene expressions in human alveolar epithelial A549 cells. Exposure of A549 cells to LPS increased SP-A mRNA synthesis in concentration and time-dependent manners without affecting SP-D mRNA production. LPS selectively enhanced translocation of transcription factor c-Jun from the cytoplasm to nuclei, but not nuclear factor kappa-B. In parallel, the DNA-binding activity of AP-1 was increased by LPS. Pretreatment of A549 cells with SP600125, an inhibitor of c-Jun N-terminal kinase, decreased c-Jun translocation, and significantly ameliorated LPS-induced SP-A mRNA production. Levels of toll-like receptor (TLR2) mRNA in A549 cells were time-dependently induced following LPS treatment. Application of TLR2 small interference (si)RNA into A549 cells significantly knocked-down the translation of this receptor, and simultaneously alleviated LPS-induced SP-A synthesis. Taken together, this study has shown that LPS selectively induces *SP-A* gene expression possibly through TLR2-mediated activation of c-Jun in human alveolar epithelial A549 cells.

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1. Introduction

Gram-negative septicemia, a complication from acute pulmonary infection, can lead to organ dysfunction or hypoperfusion abnormalities (Angus et al., 2001; Cazzola et al., 2004). Lipopolysaccharide (LPS), a Gram-negative bacterial outer membrane component, has been reported as one of the major causes of septic shock (Raetz et al., 1991; Welbourn and Yong, 1992). During pulmonary infection, surfactant proteins (SPs) participate in the physiological and pathophysiological regulation of sepsis-induced acute lung injury (Mendelson, 2000). In human lung, the LPS-induced acute pulmonary inflammation causes rapid changes in the composition of the surfactant pool and the resident cell population (Mendelson, 2000; Rooney, 2001). Pulmonary alveolar type II epithelial cells, located in the corners of the alveoli, have highly specialized functions for synthesizing, secreting, and reutilizing surfactants (Rooney, 2001). The critical function of pulmonary surfactants, a complex surface-active lipoprotein, is to reduce surface tension at the alveolar air–liquid interface, thereby preventing alveolar collapse upon expiration and allowing for normal breathing (Clements and King, 1976). Alterations in the levels of surfactant components in the lungs during inflammation are quite complex because surfactant lipids, SP-A, and SP-D appear to be regulated through host's defense mechanisms (McIntosh et al., 1996; Crouch and Wright, 2001; LeVine and Whitsett, 2001).

There are four SPs, known as SP-A, -B, -C, and -D. SP-A and -D are hydrophilic and participate in pulmonary host defense (Crouch, 1998; McCormack and Whitsett, 2002). By comparison, SP-B and SP-C are hydrophobic and contribute to the adsorption of surfactant lipids onto the surface film that lines the alveolus (McCormack and Whitsett, 2002). Previous studies have shown that transgenic mice with targeted disruptions of the *SP-A* or *SP-D* genes have increased susceptibility to infection from viral and bacterial pathogens (LeVine et al., 2000; Crouch and Wright, 2001). Levels of SP-A and SP-D can be modulated by pathogens. In acute respiratory distress syndrome associated with either Gram-positive or -negative pneumonia, inflammatory mediator-induced lung dam-

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Table 1
Effects of lipopolysaccharide on viability of A549 cells.

LPS (ng/ml)	Cell viability, OD values at 550 nm			
	1 h	6 h	16 h	24 h
0	0.62 ± 0.05	0.79 ± 0.05	0.58 ± ±0.03	0.73 ± 0.03
1	0.59 ± 0.07	0.84 ± 0.02	0.60 ± 0.02	0.71 ± 0.02
10	0.60 ± 0.07	0.82 ± 0.02	0.63 ± 0.03	0.83 ± 0.05
100	0.55 ± 0.15	0.75 ± 0.02	0.60 ± 0.02	0.78 ± 0.01
1000	0.72 ± 0.08	073 ± 0.03	0.57 ± 0.03	0.54 ± 0.01*

A549 cells were exposed to 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml lipopolysaccharide (LPS) for 1 h, 6 h, 16 h, and 24 h, respectively. Cell viability was analyzed using a colorimetric method. Each value represents mean ± SEM for $n = 6$.
* Values significantly differ from the respective control, $p < 0.05$.

age reduces the amounts of SP-A in the bronchiolar lavage fluid (Gunther et al., 1996, 2001). Regulation of *SP* gene expression by pathogens is involved via signal-transducing events. Toll-like receptor 2 (TLR2), which is a type I transmembrane protein with extracellular domains comprised largely of leucine-rich repeats and intracellular signaling domains, can be expressed in alveolar epithelial type II cells and macrophages (Droemann et al., 2003). TLR2 can transduce pathogen-triggered signals to regulate certain inflammation-related gene expressions through the activation of transcription factors (Ransone and Verma, 1990; Shaulian and Karin, 2002). Activator protein-1 (AP-1) and nuclear factor-kappa B (NFκB) are two typical transcription factors that have responded to TLR2-mediated signals and participated in the regulation of cell proliferation, differentiation, and death (Angel and Karin, 1991; Jochum et al., 2001; Shaulian and Karin, 2002).

SP-A is known to play a central role in surfactant homeostasis and function (Blau et al., 1994). George et al. (2003) reported that repeat exposure to inhaled endotoxin increased the whole lung *SP-A* gene expression, whereas SP-A protein levels in lung lavage fluid decreased (George et al., 2003). Pulmonary alveolar epithelial type II cells are the major sources of SP-A production and secretion (Rooney, 2001). However, the effects of LPS on the regulation of *SPs* gene expressions in alveolar type II epithelial cells are not well known. Therefore, this study was designed to evaluate the effects of LPS on the regulation of *SP-A* and *SP-D* gene expressions and its possible mechanisms using human lung carcinoma type II epithelium-like A549 cells as the experimental model.

2. Materials and methods

2.1. Cell culture and drug treatment

Human lung carcinoma type II epithelium-like A549 cells, purchased from the American Type Culture Collection (Rockville, MD, USA), were grown in DMEM/Ham's F-12 culture medium (Sigma Chemical, St. Louis, MO, USA) with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 2 mM L-glutamine, and 100 μg/ml streptomycin in 75 cm^2 culture flasks at 37 °C in a humidified atmosphere 5% CO_2. LPS, purchased from Sigma, was dissolved in dimethyl sulfoxide (DMSO) and sonicated to disperse large LPS aggregates as described (Kitchens et al., 2001). The concentration of DMSO in the medium was kept to less than 0.1% to avoid toxicity of this solvent to A549 cells. SP600125, an inhibitor of c-Jun N-terminal kinase, was purchased from Sigma, dissolved in DMSO, and pretreated for 1 h before LPS administration. Control cells received DMSO only.

2.2. Assay of cell viability

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Cherng et al., 2008). Briefly, A549 cells (1×10^4 cells/well) were seeded overnight in 96-well tissue

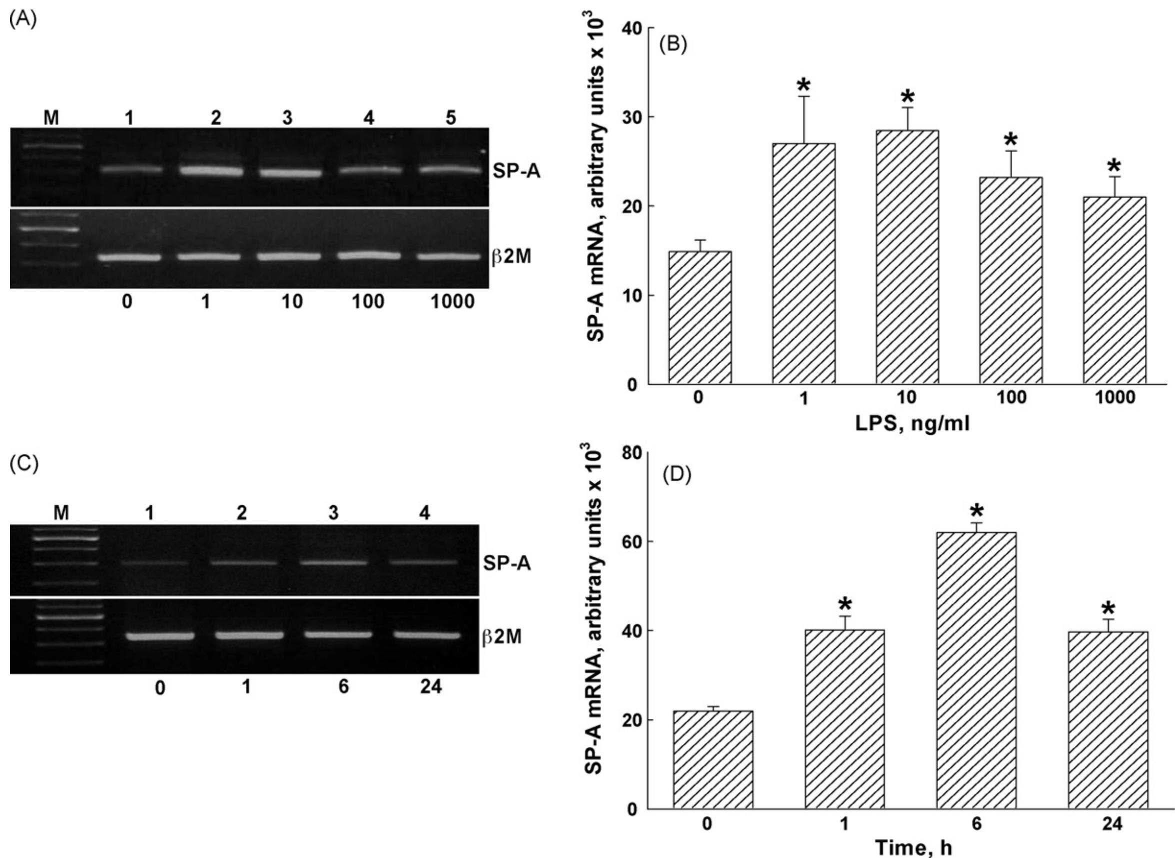

Fig. 1. Effects of lipopolysaccharide (LPS) on surfactant protein-A (SP-A) mRNA production in A549 cells. Exposure of A549 cells to 1 μg/ml, 10 μg/ml, 100 μg/ml, and 1000 μg/ml of LPS for 6 h (A and B), or to 1 μg/ml of LPS for 1 h, 6 h, and 24 h (C and D). Total RNA was prepared for RT-PCR analysis of SP-A mRNA (A and C, *top panels*). Amounts of β2M mRNA were quantified as the internal controls (A and C, *bottom panels*). These DNA bands were quantified and statistically analyzed (B and D). Each value represents the mean ± SEM for $n = 6$. The symbol, *, indicates that a value significantly ($p < 0.05$) differed from the control groups. M, DNA 100 bp marker.

culture plates. After drug treatment, A549 cells were cultured in a new medium containing 0.5 mg/ml MTT for a further 3 h. The blue formazan products in A549 cells were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

2.3. Reverse-transcription polymerase chain reaction (RT-PCR) analyses

Messenger (m)RNAs from A549 cells exposed to LPS were prepared for RT-PCR analyses of SP-A, SP-D, and β2M mRNA. The gene accession number of SP-A, SP-D, and β2M are NM 001098668.1, NM 003019.4, and NM 004048.2, respectively. Oligonucleotides for PCR analyses of SP-A, SP-D, and β2M mRNA were designed and synthesized by Clontech Laboratories (Palo Alto, CA, USA). The oligonucleotide sequences of the upstream and downstream primers for these mRNA analyses were respectively 5′-TGAAAGGGAGTTCTAGCATCTCACAGA-3′ and 5′-ACATATGCCTATGTAGGCCTGACTGAG-3′ for SP-A, 5′-AACCATTTACGGAGGCACAG-3′ and 5′-CTCTCCTGTGGGGTAGGTGA-3′ for SP-D, and 5′-GTCTACATGTCTCGATCCCACTTA A-3′ and 5′-GGTCTTTCTCTCTCATCGCGCTC-3′ for β2M. The amounts of total RNA used to synthesize SP-A, SP-D, and β2M cDNA were 200 ng, 200 ng and 50 ng, respectively. PCR was performed as 5 min at 95 °C, 32 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and followed by 72 °C for 7 min. The PCR products were loaded onto a 1.8% agarose gel containing 0.1 μg/ml ethidium bromide and electrophoretically separated. DNA bands were visualized and photographed under UV-light exposure. The length of PCR products of SP-A, SP-D, and β2M were 298 bp, 176 bp, and 335 bp, respectively. Amounts of β2M mRNA were analyzed as the internal control because its expression has been shown not to be affected by LPS (Reddy and Wilkie, 2000). The intensities of the DNA bands in the agarose gel were quantified with the aid of the UVIDOCMW version 99.03 digital imaging system (Uvtec, Cambridge, UK) as described previously (Lee et al., 2009b).

2.4. Extraction of nuclear proteins and immunodetection

The amounts of nuclear transcription factors were quantified following a previously described method (Wu et al., 2009). Briefly, after drug treatment, A549 cells were harvested and reacted on ice with ice-cold cytoplasmic extraction, reagent I and II (Pierce, Rockford, IL, USA), for 10 min and 1 min, respectively. The cell lysates were then centrifuged at 15,000 × *g* for 5 min. The nuclear pellet fraction was reacted on ice with ice-cold nuclear extraction reagent (Pierce) for 40 min. The mixtures were then centrifuged at 15,000 × *g* for 10 min, and the supernatant (nuclear extract) fraction was collected. Protein concentrations were quantified by a bicinchonic acid protein assay kit (Pierce). Nuclear proteins (50 μg/well) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The percentage of the PAGE gels was 12%. After blocking, nuclear c-Jun and NFκB were immunodetected using rabbit polyclonal antibody against mouse c-Jun and NFκB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The total c-Jun and NFκB were immunodetected as the internal standard. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

2.5. Immunoblotting analyses of TLR2, SP-A, and β-actin

Protein analyses were carried out according to a previously described method (Chang et al., 2009). After drug treatment, cell lysates were prepared in an ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl (pH 7.2), 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and 1 mM EDTA). To avoid the degradation of cytosolic proteins by proteinases, a mixture of 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate, and 5 μg/ml leupeptin was added to the RIPA buffer. Protein concentrations were quantified using a bicinchonic acid protein assay kit (Pierce). Proteins (50 μg/well) were subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The percentage of the PAGE gels was 12%. Immunodetections of TLR2 and SP-A were carried out using goat and rabbit polyclonal antibodies against mouse TLR2 and SP-A, respectively (Santa Cruz Biotechnology). Cellular β-actin protein was immunodetected using a mouse monoclonal antibody against mouse β-actin (Sigma) as the internal standard. These protein bands were quantified using a digital imaging system (UVtec).

2.6. AP-1 reporter assay

AP-1 luciferase reporter plasmids (Sratagene, La Jolla, CA, USA) and pUC18 control plasmids were transfected into A549 cells using a FuGene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) as described previously (Lee et al., 2009a). After transfection, A549 cells were exposed to LPS. Then, cells were harvested. The luciferase activity in cell lysates was measured using a dual luciferase assay sys-

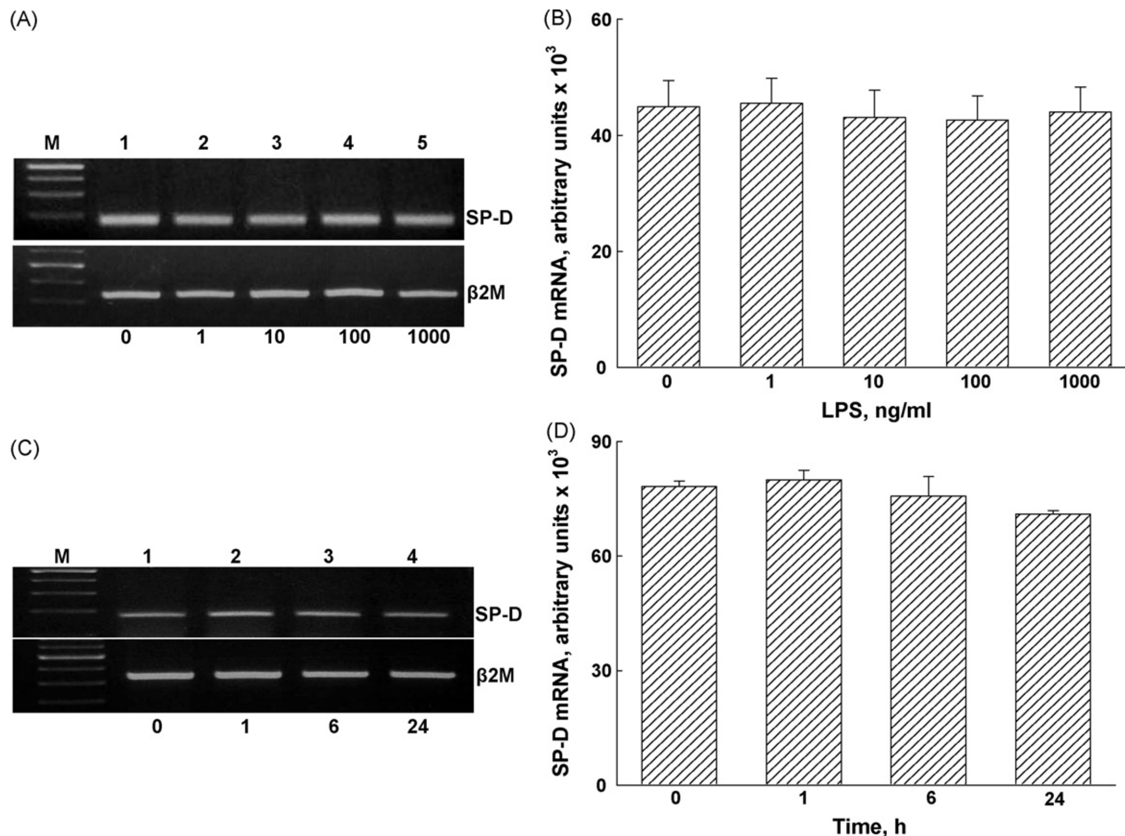

Fig. 2. Effects of lipopolysaccharide (LPS) on surfactant protein-D (SP-D) mRNA production in A549 cells. Exposure of A549 cells to 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml of LPS for 6 h (A and B), or to 1 ng/ml of LPS for 1 h, 6 h, and 24 h (C and D). Total RNA was prepared for RT-PCR analysis of SP-D mRNA (A and C, *top panels*). Amounts of β2M mRNA were quantified as the internal controls (A and C, *bottom panels*). These DNA bands were quantified and statistically analyzed (B and D). Each value represents the mean ± SEM for $n = 6$. The symbol, *, indicates that a value significantly ($p < 0.05$) differed from the respective control groups. M, DNA 100 bp marker.

tem (Promega, Madison, WI, USA). Briefly, after dispensing 100 μl of the Luciferase Assay Reagent (Promega) into luminometer tubes, the cell lysates (20 μl) were added into the tubes and vortexed briefly. The luminometer was programmed to perform a 2-s measurement delay followed by a 10-s measurement read for luciferase activity.

2.7. TLR2 knockdown

Translation of TLR2 mRNA in A549 cells was knocked-down using an RNA interference (RNAi) method following a previously described small interfering (si)RNA transfection protocol provided by Santa Cruz Biotechnology (Wu et al., 2008). TLR2 siRNA is a pool of three target-specific 20–25-nt siRNAs designed to knock-down TLR2's expression. Briefly, after culturing A549 cells in antibiotic-free DMEM/Ham's F-12 medium at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, the siRNA duplex solution, which was diluted in siRNA transfection medium (Santa Cruz Biotechnology), was added to the A549 cells. After transfection for 24 h, the medium was replaced with normal DMEM/Ham's medium, and the A549 cells were treated with LPS.

2.8. Statistical analysis

Statistical differences were considered significant when the p value of Duncan's multiple-range test was <0.05. Statistical analysis between groups over time was carried out by a two-way analysis of variance (ANOVA).

3. Results

3.1. Toxicity of LPS to A549 cells

Exposure of A549 cells to 1 ng/ml, 10 ng/ml, and 100 ng/ml LPS for 1 h, 6 h, and 24 h, respectively, did not affect cell viability (Table 1). Viability of A549 cells was not influenced following treatment with 1000 ng/ml LPS for 1 h, 6 h, and 16 h. Meanwhile, when the treated time interval reached 24 h, LPS at 1000 ng/ml caused a significant 26% decrease in viability of A549 cells (Table 1).

3.2. Induction of SP-A mRNA by LPS

RT-PCR analysis was carried out to determine the effects of LPS on SP-A mRNA production in A549 cells. In untreated A549 cells, SP-A mRNA could be detected (Fig. 1A, *top panel*, lane 1). Exposure of A549 cells to 1 ng/ml and 10 ng/ml LPS for 6 h induced SP-A mRNA synthesis (lanes 2 and 3). LPS at 100 ng/ml and 1000 ng/ml also enhanced the levels of SP-A mRNA in A549 cells, but the induction was lower than the 1 ng/ml- and 10 ng/ml-treated groups (lanes 4 and 5). Amounts of β2M mRNA were determined as the internal controls (Fig. 1A, *bottom panel*). These DNA bands were quantified, analyzed, and presented in Fig. 1B. Exposure to 1 ng/ml and 10 ng/ml LPS for 6 h significantly induced SP-A mRNA production by 3.2- and 3-fold, respectively. After treatment with 100 ng/ml and 1000 ng/ml LPS, the induced levels of SP-A mRNA in A549 cells were 1.9- and 2.2-fold, respectively (Fig. 1B).

Exposure of A549 cells to 1 ng/ml LPS for 1 h induced SP-A mRNA synthesis (Fig. 1C, *top panel*, lane 1). The LPS-induced SPA mRNA production reached a maximum at a 6-h treatment interval (lane 2). After exposure for 24 h, LPS also induced SP-A mRNA in A549 cells, but the induction decreased (lane 4). Amounts of β2M mRNA were determined as the internal controls (Fig. 1C, *bottom panel*). These DNA bands were quantified and analyzed (Fig. 1D). Treatment of A549 cells with 1 ng/ml LPS for 1 h, 6 h, and 24 h caused significant 90%, 3-fold, and 81% increases in the levels of SP-A mRNA, respectively (Fig. 1D).

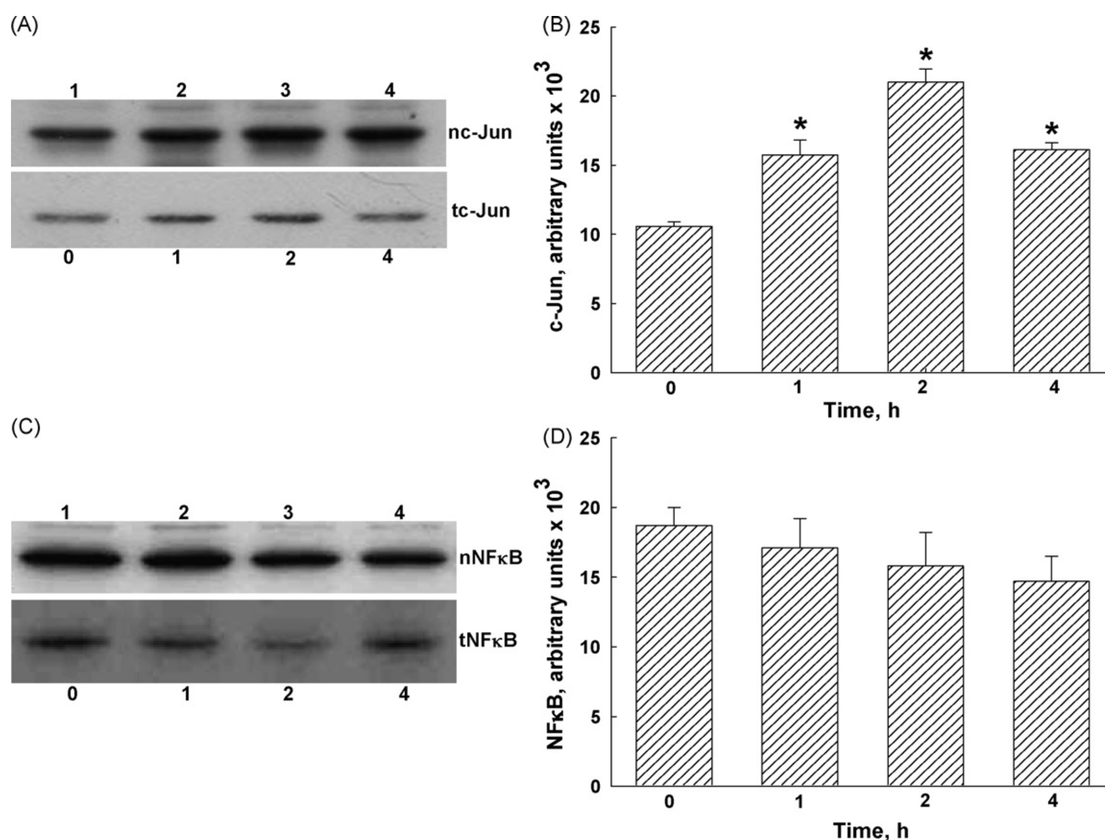

Fig. 3. Effects of lipopolysaccharide (LPS) on the levels of nuclear c-Jun and NFκ. Exposure of A549 cells to 1 ng/ml of LPS for 1 h, 2 h, and 4 h. Amounts of nuclear c-Jun (nc-Jun) and NFκB (nNFκB) were immunodetected (A and C, *top panels*). Levels of total c-Jun (tc-Jun) and NFκB (tNFκB) were analyzed as the internal standards (A and C, *bottom panels*). These protein bands were quantified and statistically analyzed (B and D). Each value represents the mean ± SEM for $n = 6$. The symbol, *, indicates that a value significantly ($p < 0.05$) differed from the control groups.

3.3. LPS did not induce SP-D mRNA expression

In the control A549 cells, SP-D mRNA could be detected (Fig. 2A, *top panel*, lane 1). Exposure of A549 cells to 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml LPS for 6 h did not change SP-D mRNA production (lanes 2–5). Amounts of β2M mRNA were quantified as the internal control (Fig. 2A, *bottom panel*). These DNA bands were quantified and analyzed (Fig. 2B). Treatment of A549 cells with 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml LPS for 6 h did not influence the levels of SP-D mRNA. After exposure to 1 ng/ml LPS for 1 h, 6 h, and 24 h, the amounts of SP-D mRNA in A549 cells were not changed (Fig. 2C, *top panel*). Levels of β2M mRNA in A549 cells were determined as the internal controls (Fig. 2C, *bottom panel*). These DNA bands were quantified and analyzed (Fig. 2D). Exposure of A549 cells to 1 ng/ml LPS for 1 h, 6 h, and 24 h did not affect SP-D mRNA expression.

3.4. LPS selectively activated c-Jun

To determine the mechanism of LPS-induced *SP-A* gene expression, the translocation of c-Jun and NFκB from the cytoplasm to nuclei was analyzed using immunodetection (Fig. 3). After exposure of A549 cells to 1 ng/ml LPS for 1 h, the levels of nuclear c-Jun were enhanced (Fig. 3A, *top panel*, lane 2). After 2- and 4-h treatment, LPS caused more translocation of c-Jun from the cytoplasm to nuclei (lanes 3 and 4). Amounts of total c-Jun in A549 cells were immunodetected as the internal control (Fig. 3A, *bottom panel*). These immunoreactive bands were quantified and analyzed (Fig. 3B). Treatment of A549 cells with 1 ng/ml LPS for 1 h, 2 h, and 4 h caused significant 45%, 2.2-fold, and 56% increases in the levels of nuclear c-Jun.

Exposure of A549 cells to 1 ng/ml LPS for 1 h, 2 h, and 4 h did not affect the levels of nuclear NFκB (Fig. 3C, *top panel*). Amounts of total NFκB in A549 cells were immunodetected as the internal control (Fig. 3C, *bottom panel*). These protein bands were quantified and analyzed (Fig. 3D). The data reveal that treatment of A549 cells with LPS for 1 h, 2 h, and 4 h did not change the levels of nuclear NFκB in A549 cells.

3.5. c-Jun activation involved in LPS-induced SP-A gene expression

Results by a reporter gene assay revealed that treatment of A549 cells with 1 ng/ml LPS for 1 h caused a 4-fold increase in the DNA-binding activity of AP-1 (Fig. 4A). After exposure for 6 h and 24 h, LPS significantly enhanced the DNA-binding activity of AP-1 by 8- and 8.7-fold, respectively. SP600125, an inhibitor of c-Jun N-terminal kinase, was applied into A549 cells to evaluate the roles of c-Jun in LPS-induced *SP-A* gene expression (Fig. 4B and C). Exposure of A549 cells to LPS induced SP-A mRNA production (Fig. 4B, *top panel*, lane 2). Alone treatment with SP600125 did not affect SP-A mRNA synthesis (lane 3). However, pretreatment of A549 cells with SP600125 could inhibit LPS-induced SP-A mRNA production (lane 4). Amounts of β2M mRNA were determined as the internal control (Fig. 4B, *bottom panel*). These DNA bands were quantified and analyzed (Fig. 4C). Exposure of A549 cells to 1 ng/ml LPS for 6 h significantly induced SP-A mRNA production by 2.5-fold. SP600125 did not influence the basal levels of SP-A mRNA in A549 cells. Meanwhile, pretreatment of A549 cells with SP600125 for 1 h completely alleviated LPS-induced SP-A mRNA synthesis (Fig. 4C).

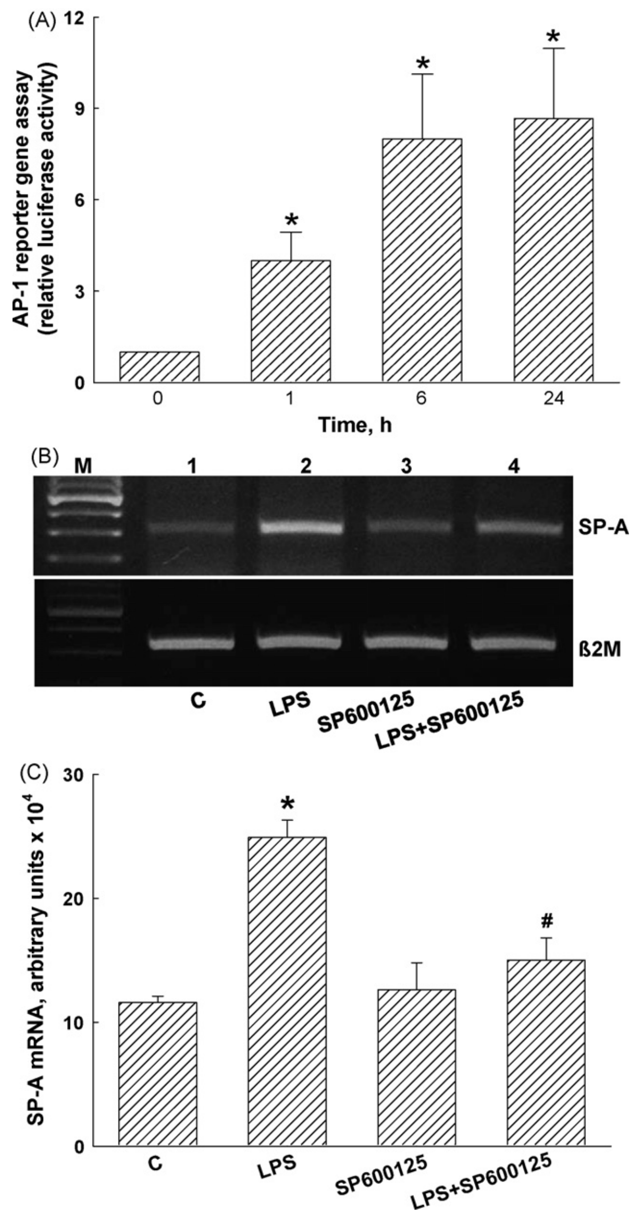

Fig. 4. Roles of AP-1 in lipopolysaccharide (LPS)-induced surfactant protein-A (SP-A) mRNA expression. A549 cells were exposed to 1 ng/ml LPS for 1 h, 6 h, and 24 h. An AP-1 reporter gene assay was carried out using AP-1 luciferase reporter plasmids (A). A549 cells were pretreated with 30 μg/ml SP600125, an inhibitor of c-Jun N-terminal kinase, for 1 h, and then exposed to 1 ng/ml LPS for another 6 h. Total RNA was prepared for RT-PCR analysis of SP-A mRNA (B, *top panel*). Amounts of β2M mRNA were quantified as the internal controls (*bottom panel*). These DNA bands were quantified and statistically analyzed (C). Each value represents the mean ± SEM for $n = 6$. The symbol, *, indicates that a value significantly ($p < 0.05$) differed from the control groups. C, control; M, DNA 100 bp marker.

3.6. TLR2 participating in LPS-induced SP-A gene expression

In untreated A549 cells, TLR2 mRNA could be detected (Fig. 5A, *top panel*, lane 1). Exposure of A549 cells to 1 ng/ml LPS for 1 h induced TLR2 mRNA production (lane 2). After treating for 6 h and 24 h, the levels of TLR2 mRNA in A549 cells were induced by LPS (lanes 3 and 4). Amounts of β2M mRNA in A549 cells were determined as the internal control (Fig. 5A, *bottom panel*). These DNA bands were quantified and analyzed (Fig. 5B). Exposure of A549 cells to LPS for 1 h, 6 h, and 24 h caused significant 48%, 2.4-fold, and 52% increases in the levels of TLR2 mRNA.

Translation of TLR2 was knocked-down by RNA interference to evaluate the role of this membrane receptor in LPS-induced *SP-A* gene expression (Fig. 5C and D). Application of TLR2 siRNA into A549 cells for 24 h and 48 h decreased the levels of this recep-

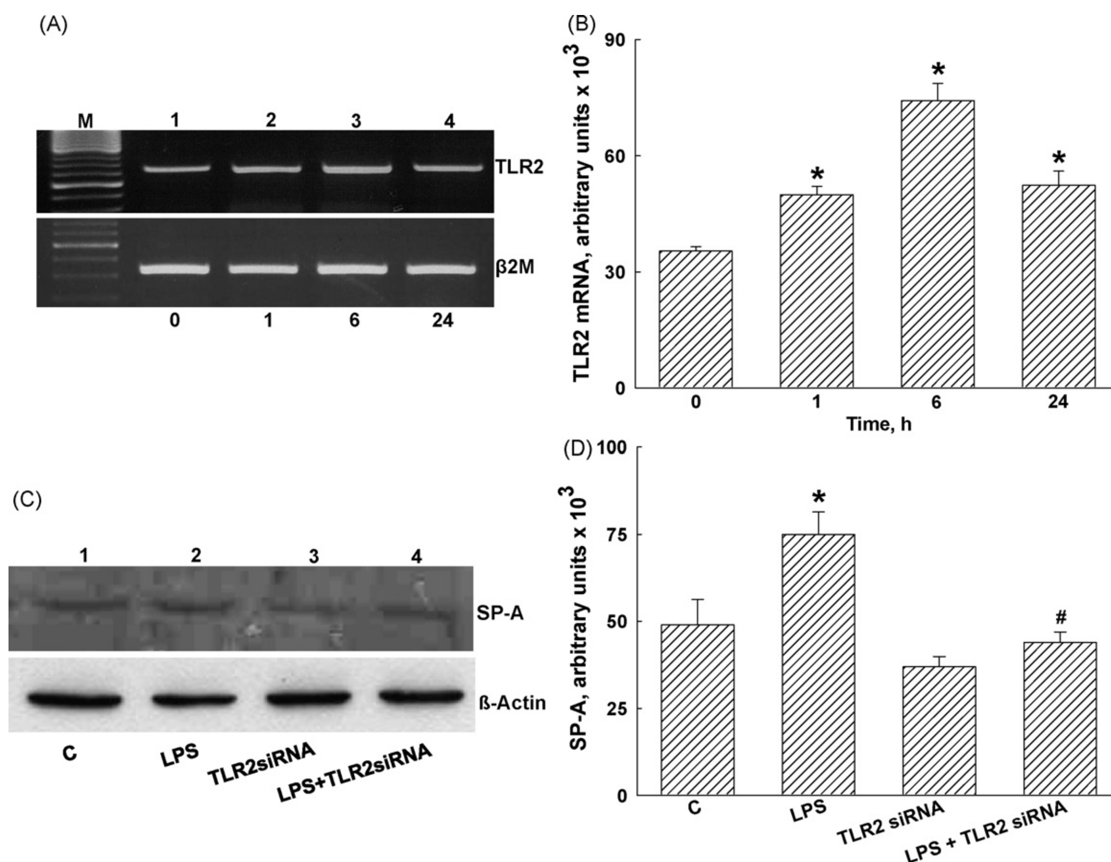

Fig. 5. Effects of lipopolysaccharide (LPS) on toll-like receptor 2 (TLR2) mRNA production, and the role of TLR2 in LPS-enhanced surfactant protein-A (SP-A). A549 cells were treated with 1 ng/ml LPS for 1 h, 6 h, and 24 h (A). Total RNA was prepared for RT-PCR analysis of TLR2 mRNA (*top panel*). Amounts of β2M mRNA were quantified as the internal controls (*bottom panel*). These DNA bands were quantified and statistically analyzed (B). A549 cells were exposed to TLR2 small interference (si)RNA, LPS, and a combination of TLR2 siRNA and LPS for 24 h (C). Amounts of SP-A in A549 cells were immunodetected (*top panel*). Levels of β-actin were determined as the internal controls (*bottom panel*). These protein bands were quantified and statistically analyzed (D). Each value represents the mean ± SEM for $n = 6$. The symbol, *, indicates that a value significantly ($p < 0.05$) differed from the control groups. C, control; M, DNA 100 bp marker.

tor in a time-dependent manner (data not shown). Treatment of A549 cells with LPS increased SP-A synthesis (Fig. 5C, *top panel*, lane 2). Application of TLR2 siRNA into A549 cells did not affect SP-A production (lane 3). Meanwhile, application of TLR2 siRNA decreased LPS-enhanced SP-A production (lane 4). Amounts of β-actin in A549 cells were immunodetected as the internal control (Fig. 5C, *bottom panel*). These immunorelated protein bands were quantified and analyzed (Fig. 5D). Exposure of A549 cells to LPS significantly increased the levels of SP-A by 79%. Co-treatment with TLR2 siRNA and LPS completely ameliorated LPS-enhanced SP-A production (Fig. 5D).

4. Discussion

This study has shown that LPS at a clinically relevant concentration of 1 ng/ml induces *SP-A* gene expression in human lung carcinoma type II epithelium-like A549 cells. The concentrations of LPS used in *in vitro* and *in vivo* studies were about 1 μg/ml and 1 mg/kg body weight, respectively (Millar and Thiemermann, 1997; Wu et al., 2008). Bysani et al. (1990) reported that the plasma concentration of LPS in a patient with fatal *Klebsiella pneumoniae* sepsis was 25 ng/ml (Bysani et al., 1990). In this study, we demonstrated that LPS even at 1 ng/ml could induce SP-A mRNA and protein expression. LPS selectively induces SP-A expression in A549 cells. This result is similar to a previous study, which showed that SP-A is a better indicator than SP-D for detecting the severity of the acute lung injury and its clinical outcome (Dulkerian et al., 1996; Cheng et al., 2003). Decreases in alveolar SP-A have been reported in patients with bacterial pneumonia (Baughman et al., 1993; Gunther et al., 1996). Therefore, induction of SP-A in pulmonary type II epithelial cells by LPS at a clinically relevant concentration may be used as an indicator in response to the process of endotoxin-caused septic shock and acute lung injury.

c-Jun activation can be involved in LPS-induced *SP-A* expression. After exposure to LPS, the levels of nuclear c-Jun were time-dependently augmented. c-Jun is a critical member of transcription factor AP-1 (Angel and Karin, 1991). In response to the stimulus, c-Jun N-terminal kinase is an upstream kinase for phosphorylating AP-1 (Ransone and Verma, 1990; Angel and Karin, 1991). Our unpublished study showed that when the activity of c-Jun N-terminal kinase was suppressed by its specific inhibitor SP600125, the LPS-enhanced c-Jun translocation from the cytoplasm to the nuclei was consequently reduced. Thus, LPS possibly activates c-Jun through the stimulation of c-Jun N-terminal kinase phosphorylation. The present results reveal that application of SP600125 into A549 cells significantly inhibited LPS-induced SP-A mRNA production. In the promoter region of the *SP-A* gene, specific DNA-binding motifs of AP-1 can be found (Miakotina and Snyde, 2004). Jun and Fos are two critical components for constructing AP-1 heterodimers (Hess et al., 2004). A reporter gene assay showed that LPS time-dependently increased the DNA-binding activity of AP-1. Therefore, the LPS-induced *SP-A* gene expression may be due to the increases in translocation and transactivation of AP-1 in A549 cells following exposure to the endotoxin.

The LPS-induced *SP-A* gene expression occurs possibly via the TLR2-dependent pathway. Muzio et al. (2000) reported that LPS could not induce TLR2 expression but rather, the selective stimulation of TLR-3 production in dendritic cells. In human monocytes, LPS was demonstrated to induce TLR2 expression (Flo et al., 2001). This study further showed that LPS at a clinically relevant concentration of 1 ng/ml could enhance TLR2 mRNA and protein syntheses in human pulmonary type II epithelium-like A549 cells. In response to LPS stimulation, AP-1 is demonstrably activated through TLR-dependent mechanisms (Ransone and Verma, 1990; Shaulian and Karin, 2002). In the human lung, TLR2 is detected in alveolar type II epithelial cells and alveolar macrophages (Droemann et al., 2003). Thus, LPS increases the translocation of AP-1 from the cytoplasm to nuclei, possibly through TLR2-dependent signal-transducing events. Furthermore, application of TLR2 siRNA caused significant downregulation in the LPS-induced SP-A protein synthesis. Therefore, LPS induces *SP-A* gene expression via upregulating the TLR2-dependent translocation of AP-1 from the cytoplasm to nuclei and its transactivation activity.

In conclusion, this study shows that exposure of A549 cells to LPS at clinically relevant concentrations did not affect cell viability but selectively induced SP-A expression at protein and mRNA levels without affecting SP-D synthesis. In parallel with increases in SP-A expression, LPS increased the translocation of c-Jun from the cytoplasm to the nuclei and the transactivation activity of AP-1. Treatment of A549 cells with SP600125, an inhibitor of c-Jun N-terminal kinase, ameliorated LPS-induced SP-A mRNA production. Exposure to LPS time-dependently induced TLR2 mRNA synthesis. Application of TLR2 siRNA decreased translation of this membrane receptor, and simultaneously alleviated LPS-enhanced TLR2 production. Therefore, we suggest that LPS selectively induces SP-A gene expression in pulmonary epithelial A549 cells possibly through a TLR2-dependent activation of c-Jun.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

Angel, P., Karin, M., 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta 1072, 129–157.
Angus, D.C., Linde-Zwirble, W.T., Lidicker, J., Clermont, G., Carcillo, J., Pinsky, M.R., 2001. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit. Care Med. 29, 1303–1310.
Baughman, R.P., Sternberg, R.I., Hull, W., Buchsbaum, J.A., Whitsett, J., 1993. Decreased surfactant protein A in patients with bacterial pneumonia. Am. Rev. Respir. Dis. 147, 653–657.
Blau, H., Riklis, S., Kravtsov, V., Kalina, M., 1994. Secretion of cytokines by rat alveolar epithelial cells: possible regulatory role for SP-A. Am. J. Physiol. 266, L148–L155.
Bysani, G.K., Shenep, J.L., Hildner, W.K., Stidham, G.L., Roberson, P.K., 1990. Detoxification of plasma containing lipopolysaccharide by adsorption. Crit. Care Med. 18, 67–71.
Cazzola, M., Page, C.P., Matera, M.G., 2004. Alternative and/or integrative therapies for pneumonia under development. Curr. Opin. Pulmonary Med. 10, 204–210.
Chang, H.C., Chen, T.L., Chen, R.M., 2009. Interruption of hepatocyte cytoskeletons by ketamine occurs through suppression of calcium mobilization and mitochondrial function. Drug Metab. Dispos. 37, 24–31.
Cheng, I.W., Ware, L.B., Greene, K.E., Nuckton, T.J., Eisner, M.D., Matthay, M.A., 2003. Prognostic value of surfactant proteins A and D in patients with acute lung injury. Crit. Care Med. 31, 20–27.
Cherng, Y.G., Chang, H.C., Lin, Y.L., Kuo, M.L., Chiu, W.T., Chen, R.M., 2008. Apoptotic insults to human chondrocytes induced by nitric oxide are involved in sequential events, including cytoskeletal remodeling, phosphorylation of mitogen-activated protein kinase kinase kinase-1, and Bax-mitochondria-mediated caspase activation. J. Orthop. Res. 26, 1018–1026.
Clements, J.A., King, R.J., 1976. In: Crystal, R.G. (Ed.), The Biochemical Basis of Pulmonary Function. Dekker, New York, pp. 363–387.
Crouch, E., Wright, J.R., 2001. Surfactant proteins A and D and pulmonary host defense. Annu. Rev. Physiol. 63, 521–554.
Crouch, E.C., 1998. Collectins and pulmonary host defense. Am. J. Respir. Cell Mol. Biol. 19, 177–201.
Droemann, D., Goldmann, T., Branscheid, D., Clark, R., Dalhoff, K., Zabel, P., Vollmer, E., 2003. Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. Histochem. Cell. Biol. 119, 103–108.
Dulkerian, S.J., Gonzales, L.W., Ning, Y., Ballard, P.L., 1996. Regulation of surfactant protein D in human fetal lung. Am. J. Respir. Cell Mol. Biol. 15, 781–786.
Flo, T.H., Halaas, O., Torp, S., Ryan, L., Lien, E., Dybdahl, B., Sundan, A., Espevik, T., 2001. Differential expression of toll-like receptor 2 in human cells. J. Leukoc. Biol. 69, 474–481.
George, C.L., White, M.L., O'Neill, M.E., Thorne, P.S., Schwartz, D.A., Snyder, J.M., 2003. Altered surfactant protein A gene expression and protein metabolism associated with repeat exposure to inhaled endotoxin. Am. J. Physiol. 285, L1337–L1344.
Gunther, A., Ruppert, C., Schmidt, R., Markart, P., Grimminger, F., Walmrath, D., Seeger, W., 2001. Surfactant alteration and replacement in acute respiratory distress syndrome. Respir. Res. 2, 353–364.
Gunther, A., Siebert, C., Chmidt, R., Ziegler, S., Grimminger, F., Yabut, M., Temmesfeld, B., Walmrath, D., Morr, H., Seeger, W., 1996. Surfactant alterations in severe pneumonia, acute respiratory distress syndrome, and cardiogenic lung edema. Am. J. Respir. Crit. Care Med. 153, 176–184.
Hess, J., Angel, P., Schorpp-Kistner, M., 2004. AP-1 subunits: quarrel and harmony among siblings. J. Cell Sci. 117, 5965–5973.
Jochum, W., Passegue, E., Wagner, E.F., 2001. AP-1 in mouse development and tumorigenesis. Oncogene 20, 2410–2412.
Kitchens, R.L., Thompson, P.A., Viriyakosol, S., O'Keefe, G.E., Munford, R.S., 2001. Plasma CD14 decreases monocyte responses to LPS by promoting the transfer of cell-bound LPS to plasma lipoproteins. J. Clin. Invest. 108, 485–493.
Lee, C.J., Tai, Y.T., Lin, Y.L., Chen, R.M., 2009a. Molecular mechanisms of propofol-involved suppression of nitric oxide biosynthesis and *inducible nitric oxide synthase* gene expression in lipopolysaccharide-stimulated macrophage-like Raw 264.7 cells. Shock, doi:10.1097/SHK.0b013e3181a6eaf5.
Lee, S.T., Wu, T.T., Yu, P.Y., Chen, R.M., 2009b. Apoptotic insults to human HepG2 cells induced by S-(+)-ketamine occurs through activation of a Bax-mitochondria-caspase protease pathway. Br. J. Anaesth. 102, 80–89.
LeVine, A.M., Whitsett, J.A., 2001. Pulmonary collectins and innate host defense of the lung. Microbes Infect. 3, 161–166.
LeVine, A.M., Whitsett, J.A., Gwozdz, J.A., Richardson, T.R., Fisher, J.H., Burhans, M.S., Korfhagen, T.R., 2000. Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung. J. Immunol. 165, 3934–3940.
McCormack, F.X., Whitsett, J.A., 2002. The pulmonary collectins SP-A and SP-D orchestrate innate immunity in the lung. J. Clin. Invest. 109, 707–712.
McIntosh, J.C., Swyers, A.H., Fisher, J.H., Wright, J.R., 1996. Surfactant proteins A and D increase in response to intratracheal lipopolysaccharide. Am. J. Respir. Cell Mol. Biol. 15, 509–519.
Mendelson, C.R., 2000. Role of transcription factors in fetal lung development and surfactant protein gene expression. Annu. Rev. Physiol. 62, 875–915.
Miakotina, O.L., Snyde, J.M., 2004. Signal transduction events involved in TPA downregulation of SP-A gene expression. Am. J. Physiol. 286, L1210–L1219.
Millar, C.G., Thiemermann, C., 1997. Intrarenal haemodynamics and renal dysfunction in endotoxaemia: effects of nitric oxide synthase inhibition. Br. J. Pharmacol. 121, 1824–1830.
Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P., Mantovani, A., 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. 164, 5998–6004.
Raetz, C.R., Ulevitch, R.J., Wright, S.D., Sibley, C.H., Ding, A., Nathan, C.F., 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. FASEB J. 5, 2652–2660.
Ransone, L.J., Verma, I.M., 1990. Nuclear proto-oncogenes *fos* and *jun*. Annu. Rev. Cell Biol. 6, 539–557.
Reddy, N.R., Wilkie, B.N., 2000. Quantitation of porcine cytokine and beta 2-microglobulin mRNA expression by reverse transcription polymerase chain reaction. J. Immunol. Methods 233, 83–93.
Rooney, S.A., 2001. Regulation of surfactant secretion. Comp. Biochem. Physiol. 129, 233–243.
Shaulian, E., Karin, M., 2002. AP-1 as a regulator of cell life and death. Nat. Cell Biol. 4, E131–E136.
Welbourn, C.R.B., Yong, Y., 1992. Endotoxin, septic shock and acute lung injury: neutrophils, macrophages, and inflammatory mediators. Br. J. Surg. 79, 998–1003.

Wu, G.J., Chen, T.L., Chang, C.C., Chen, R.M., 2009. Toll-like receptor 2-mediated nuclear factor-kappaB activation pathway contributes to ketamine-involved suppression of tumour necrosis factor-α expression in lipoteichoic acid-stimulated macrophages. Chem.-Biol. Interact. 180, 465–471.

Wu, G.J., Chen, T.L., Ueng, Y.F., Chen, R.M., 2008. Ketamine inhibits tumor necrosis factor-α and interleukin-6 gene expressions in lipopolysaccharide-stimulated macrophages through suppression of toll-like receptor 4-mediated c-Jun N-terminal kinase phosphorylation and activator protein-1 activation. Toxicol. Appl. Pharmacol. 228, 105–113.