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# Temporal changes of pulmonary surfactant protein D in young rats with acute lung injury induced by lipopolysaccharide

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**Abstract: Objective** Pulmonary surfactant protein-D (SP-D) is regarded as a valuable biomarker in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), but the alterations of SP-D in lung tissues in the early course of ALI remain unknown. This study was designed to explore the temporal fluctuations of SP-D and SP-D mRNA in young rats with ALI induced by lipopolysaccharide (LPS), as well as the alterations of ultrastructures of alveolar type Ⅱ (AT Ⅱ) cells. **Methods** Rat ALI models were established by intraperitoneal injection of LPS (4 mg/kg). The rats were sacrificed at 6, 12, 24, 36, 48 and 72 hrs after LPS injection (8 rats each time point). Western blot and RT-PCR were employed to detect the contents of SP-D and SP-D mRNA in lung tissues. The ultrastructures of AT Ⅱ cells were studied with transmission electron microscopy. **Results** Both SP-D mRNA and SP-D levels decreased after 12 hrs of LPS administration. The SP-D mRNA level reached a nadir at 24-36 hrs, but the SP-D level was reduced to its nadir by 48 hrs after LPS administration. LPS resulted in the alterations of lamellar bodies (LBs) in size (multilamellar forms), density (vacuole-like deformity) and number. The alterations of ultrastructures of AT Ⅱ cells were most significant at 48 hrs. The clinical symptoms of ALI rats were most severe at 48 hrs. **Conclusions** The alterations of the SP-D level were time-dependent in the early course of LPS-induced ALI. The lowest level of SP-D occurred at 48 hrs while severe multideformities of AT Ⅱ cells were presented. A decreased level of SP-D in the lungs in the early stage of ALI may be associated with a worse clinical outcome. [**Chin J Contemp Pediatr**, **2005**, **7**(**6**):**483 – 488**]

**Key words:** Lipopolysaccharide; Acute lung injury; Pulmonary surfactant protein D; Alveolar type Ⅱ cells; Lamellar body; Rats

## 肺表面活性物质蛋白-D 在脂多糖诱导的急性肺损伤幼鼠中的时序变化

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[**摘 要**] **目的** 肺表面活性物质蛋白 D(SP-D)被认为是急性肺损伤(ALI)和急性呼吸窘迫综合征(ARDS)有价值的生物指标,但在急性肺损伤早期,肺组织 SP-D 的变化特征仍不清楚。该研究旨在探究脂多糖(LPS)诱导的 SD 幼鼠急性肺损伤时 SP-D,SP-D mRNA 的时序变化及肺泡Ⅱ型上皮细胞及板层小体的超微结构的变化。**方法** 腹腔内注射 LPS 建立急性肺损伤模型。注射后 6,12,24 ,36,48,72 h 各处死 8 只大鼠。Western blot 和 RT-PCR 方法测定肺组织 SP-D 和 SP-D mRNA 的含量。透射电子显微镜研究肺泡Ⅱ型上皮细胞超微结构的变化。**结果** LPS 注射 12 h 后 SP-D 和 SP-D mRNA 含量均开始下降。SP-D mRNA 于注射 LPS 后 24 ~36 h 降到最低。SP-D 在 48 h 达最低点。透射电镜显示急性肺损伤组板层小体出现多样变形,特别是在注射后 48 h。LPS 导致板层小体的体积增大、数量减少,伴有大量空泡样变。**结论** 在 LPS 诱导的急性肺损伤的早期 SP-D 的波动变化呈时间依赖性。肺组织 SP-D 在 48 h 时水平最低,此时伴有肺泡Ⅱ型上皮细胞严重的多形性变。在 ALI 发病初期,肺组织低水平的 SP-D 与较差的临床预后有关。 [**中国当代儿科杂志,2005, 7(6):483 – 488**]

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Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common. They result in a case fatality rate of 40%-70%[1,2,3]. ALI is the early stage of ARDS[1,4]. Pulmonary surfactant protein D (SP-D) is synthesized in alveolar type Ⅱ (ATⅡ) cells and is secreted into alveoli and conducting airways. SP-D plays an important role in innate immunity [5,6] determined by its domain structures and configuration. The basic subunit of SP-D consists of four major domains: a short cysteine-containing NH2-terminal cross-linking domain (N), a triple helical collagen domain of variable length, a trimeric coiled-coil linking domain and a carboxyl-terminal which is C-type lectin carbohydrate recognition domain (CRD). SP-D is predominantly assembled as dodecamers consisting of four homotrimeric subunits (12 chains) with relatively long triple helical arms [7,8]. SP-D dodecamers can self-associate at their amino-termini to form much more highly ordered, stellate multimers with peripheral arrays of trimeric CRDs [7,9,10] which called SP-D multimers. SP-D binds to lipopolysaccharide (LPS) isolated from a variety of gram-negative bacteria [11,12]. SP-D was identified as the major *E. coli* binding protein [11]. The LPS′s core domain of *E. coli* has been identified as a major ligand for SP-D of rats or humans [12].

SP-D is regarded as a valuable biomarker in ALI [13] and ARDS. SP-A and SP-D levels were increased in bronchiolar alveolar lavage fluid (BALF) in response to intratracheal LPS, but SP-D levels in lung tissues have not been reported [14]. SP-D levels in lung homogenate, BALF and serum of rats instilled with bleomycin increased, and SP-D levels also elevated in rats treated intrabronchially with hydrochloric acid [13]. The increased levels of plasma of SP-D in the early course of ALI/ARDS were associated with the higher risk of death [15]. The higher plasma SP-A concentrations with little SP-D at the onset of ALI had close relationship with a worse clinical outcome [16]. So far, the association of SP-D with ALI has not been elucidated. Though SP-D plays an important role in the defense of host innate immunity as well as a main component of pulmonary surfactant, the behavior of SP-D against LPS is still unknown. This study was to explore temporal fluctuations of SP-D protein and SP-D mRNA levels and the micromorphological changes of ATⅡ cells in young rats with LPS-induced ALI.

## Materials and methods

### Materials

LPS was isolated from *E. coli* (O55:B5, Sigma). Goat anti-rat SP-D polyclonal antibodies were purchased from Santa Cruz, Biotechnology, Inc.. Total mRNA extract reagents were purchased from Huamei Bioengineering Inc. RT-PCT reagents were purchased from TakaRa.

### Subjects and ALI model

Seventy pathogen-free 21-day-old Sprague-Dawley rats weighing 44-61 g, provided by the Animal Department of China Medical University with the permission of Ethics Committee, were assigned into Control and ALI groups. Rats in the ALI group were intraperitoneal injected with 4 mg/kg LPS [17] in order to induce acute lung injury, while the control rats were injected with normal saline. The AIL rats were anesthetized with 10% chloral hydrate (4 mL/kg) and were sacrificed by incising the abdominal aorta at 6, 12, 24, 36, 48 and 72 hours after LPS injection (8 rats each time point). Diarrhea, cyanosis and dyspnea occurred in the LPS group but the control rats had no such symptoms. The body weights of the surviving rats in the LPS group were reduced when compared with those of the control groups.

### Left lung homogenate

The left lung tissues were placed into a homogenizing buffer (50 mM Tris · HCl, pH 7.5, containing 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 2.5 mM N-ethylmaleimide) at a defined ratio of 1 g of lung tissue to 9 mL of homogenizing buffer. The lung tissues were homogenized on ice with a Polytron (Brinkman Instruments, Westbury, NY). The lung homogenate was sonicated on ice (20 s, 5 times), and spun at 300 g for 5 minutes to sediment tissue debris.

### RT-PCR

Total RNA from whole lung tissues was isolated by ultracentrifugation according to the instructions of total RNA extraction. The RNA concentration was calculated by the A260 value measured with the ultraviolet spectrophotometer. The purity of RNA was judged by the ratio of A260 to A280. The reverse transcription reaction system included (20 μL): RNA 1 μg, 2 ×

buffer 10 μL, $MgSO_4$ (25 mM) 4 μL, dNTPs (10 mM) 1 μL, reversed transcriptive enzyme (22 U/μL) 1 μL, Oligo-dT15 (50 μM) 1μL, RNase-Inhibitor (40 U/μL) 0.5 μL, dd $H_2O$ 0.5 μL. cDNA was produced under the following conditions: 65 ℃ 1 minute → 30 ℃ 5 minutes ( the temperature increased regularly from 30 ℃ to 65 ℃ in 30 minutes ) →65 ℃ 30 minutes → 98 ℃ 5 minutes → 5 ℃ 5 minutes. The amplified fragment of SP-D was 285 bp. The forward primer for SP-D was 5′-GAA TCA AAG GCG AAA GTG G-3′, and reverse primer was 5′-TGC TGT GGG CTG TGA CGA-3′. The reaction mixture included (50 μL) cDNA 3 μL, 10 × buffer 5 μL, dNTPs (10mM) 1 μL, Taq-enzyme (5 U/μL) 0.5 μL, forward primer (10 μM) 0.2 μL and reverse primer (10μM) 0.2 μL, dd $H_2O$ to 50 μL. The PCR was conducted as following: 94 ℃ 3 minutes → 94 ℃ 45 seconds → 55 ℃ 1 minutes → 72 ℃ 1 minutes → 72 ℃ 7 minutes. After 35 cycles, it was extended at 72 ℃ for 5 minutes. The 2% agarose gel electrophoresis was employed to detect the amplified products. The density of bands was assessed and the content of SP-D mRNA was expressed as the ratio to β-actin.

**Total protein assay**

Protein concentrations in lung homogenate were measured by Lowry′s method in order to adjust the samples′ concentrations identically before electrophoresis.

**Western blot analysis**

The 20 μL of samples from each group were separated by 8% SDS-polyacrylamide gel electrophoresis with 120 voltage for 1.5 hours. Gels were blotted onto nitrocellulose membranes ( Millipore Corporation ) between two sheets of 3 MM filter paper ( Whatman, Clifton, NJ) in a transfer tank using 192 mM glycine, 20% methanol, 25 mM Tris-HCl, pH 8.0, under constant 50 voltage 2 hours, with stirring at 4℃ for 3 hours. Transfer was verified by staining the gel and the nitrocellulose membranes. The membranes were blocked for 2 hours in 5% low-fat milk in phosphate-buffered saline, and then washed three times for 10 minutes in PBS-T. and Membranes were incubated in goat anti-rat SP-D 1:500 in 1 % PBS-T for 1 hour, washed three times for 10 minutes each in 1 % PBS-T, and then detected with donkey anti-goat serum 1:2000 in 1 % PBS-T (Santa Cruz). Membranes were again washed three times in PBS-T and then incubated three times for 10 minutes each in alkalescence phosphatase buffer solution. β-Naphthyl acid phosphate and o-Dianisidine, Tetrazotized were used to dye the membranes. The membranes were scanned and photographed. Pictures of SP-D were t quantified by using the FlourChem Digital Imaging System V 2.0 ( Alpha Innotech Corporation ). The statistical data derived from integrated density values (IDVs) were computed with SPSS 11.5 software and were denoted in one in a million.

**Transmission electron microscopy**

One $mm^3$ of samples were obtained from the lower part of right lung and were fixed twice with 2.5% glutaraldehyde. The pellet was rinsed repeatedly in PBS. Before being embedded in araldite ( Epon812, Japan ), the samples were post-fixed in 2% OsO4 (England) in PBS for 1 hour and then dehydrated in concentration grading alcohol solutions. Slices cut with LKB Super-thin cutting machine (Sweden) were double stained with natrium and lead. The samples were observed under a JEM 100CX-Ⅱ transmission electron microscope (Japan).

**Statistical analysis**

Statistical analysis was performed with ANOVA by SPSS 11.5 software. Data were expressed as $\bar{x} \pm s$.

## Results

**Temporal changes of SP-D mRNA levels in LPS-induced ALI**

Levels of SP-D mRNA and SP-D in ALI rats changed dramatically after LPS injection. The temporal changes of SP-D mRNA and SP-D after LPS injection were different. The SP-D mRNA level gradually decreased but the SP-D level increased within 6 hours after LPS injection compared with that of the Control group. They both remained stable between 6 and 12 hrs. After 12 hours the SP-D mRNA level decreased sharply and reached a nadir at 24 hours. It remained the lowest level for 12 hours. The SP-D level was reduced at 24 hours but reached a nadir at 48 hours. The SP-D mRNA level began to increase at 48 hours, being 12 hours earlier than the increase of SP-D level ( Figure 1).

**Ultrastructural changes of AT Ⅱ cells**

The ultrastructural changes of AT Ⅱ cells in the lung tissues were studied using the transmission electron microscopy. LPS resulted in the alterations of LBs in size ( multilamellar forms ), density ( vacuole-like deformity) and number ( Figure 2).

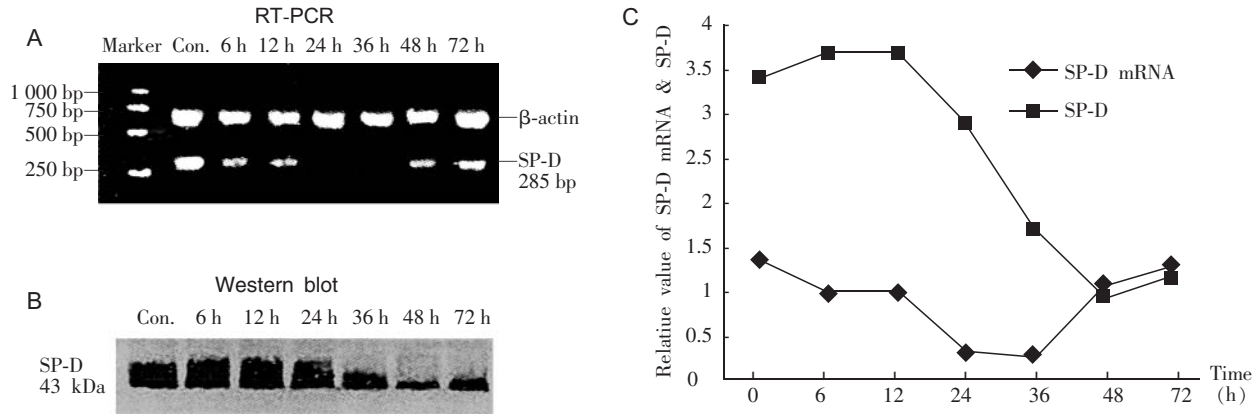


**Figure 1 Temporal changes of SP-D protein and mRNA levels in LPS-induced ALI.** The changes of the SP-D mRNA level were time-dependent. It was significantly reduced from 6 hrs after LPS injection and fell to the lowest levels between 24 and 36 hrs, then rose up greatly at 48 and 72 hrs after LPS injection. The SP-D level increased slightly at 6 and 12 hrs post-LPS injection, but decreased at 24 hrs. It was reduced to a nadir by 48 hrs. The levels of SP-D mRNA and SP-D changed in an opposite way within 6 hrs of LPS injection and remained stable between 6 and 12 hrs. After 12 hrs of LPS injection they changed in a same way. ( con: Control group)

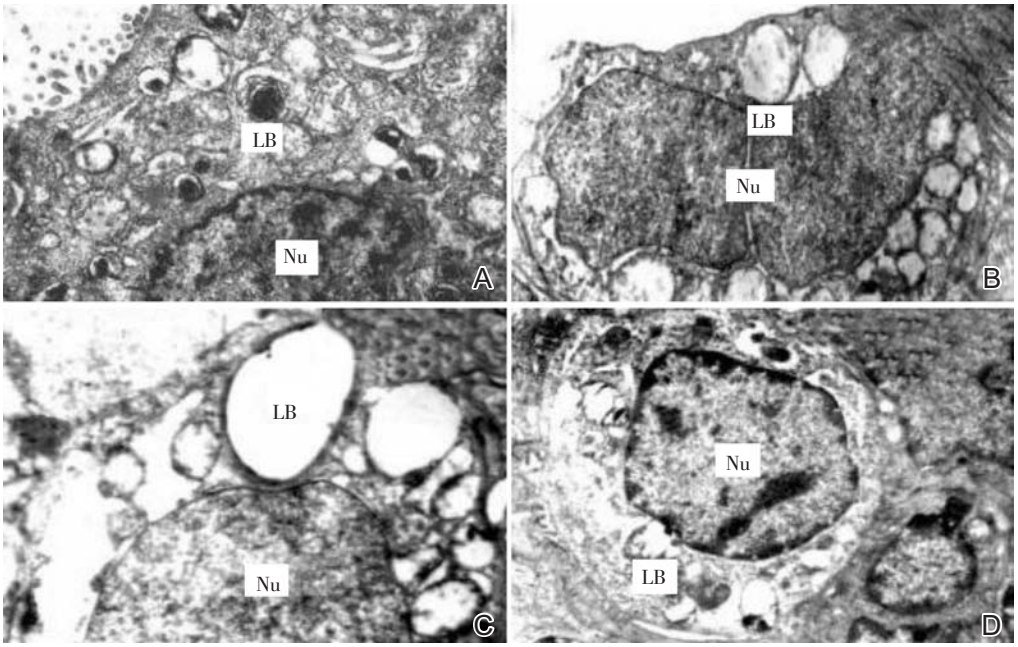


**Figure 2 Ultrastructural changes of AT Ⅱ cells.** **A** ( ×10 000): Ultrastructures in normal lungs. The LBs were presented clearly with the round shape and normal density, and the villi were obvious. **B** ( ×7 200): At 24 hrs after LPS administration, the number of LBs increased. Two nuclei were ringed by the LBs with attenuated density. Villi disappeared. **C** ( ×10 000): At 48 hrs after LPS administration, LBs revealed in multilamellar forms, and the gigantic LB presented with vacuole-like deformity. The density of LBs was attenuated significantly in this period. The number of LBs was reduced as compared with that of 24 hrs group. **D** ( ×7 200): At 72 hrs after LPS administration, the remnant of LBs shrank in size and the number of LBs decreased prominently, and LBs scattered around the nucleus. The shapes and sizes of LBs were more homogeneous than that of 48 hrs group. The density of LBs slightly increased compared with that of 48 hrs group. Villi were not found. (Note: LB: Lamellar body; Nu: Nucleus)

## Discussion

SP-D synthesis is greatly dominated by the expression of SP-D mRNA. This study showed that the changes of SP-D mRNA and SP-D expression did not run synchronously. The changes of SP-D expression occurred later (12-24 hrs) than those of SP-D mRNA expression. It may be deduced that the time from SP-D mRNA expression to SP-D production [18] must have been consistent with the hysteresis time of SP-D expression.

According to the appearance of the curves in Figure 1C, the process for the reaction of SP-D mRNA and SP-D against LPS was divided into three stages in order to expatiate the mechanism clearly. The first stage referred to the first 12 hours after LPS injection. The second one lasted from 12 to 48 hours with the lowest expression of SP-D. The third stage occurred from 48 to 72 hours.

The expression of SP-D was greatly influenced by the inflammatory mediators. In the first and second stages, the down-regulation of SP-D mRNA expression resulted from the overproduction of TNF-α and IL-6 as well as other cytokines. LPS increased significantly lung TNF-α[19] and IL-6[20] at 1 and 6 hours. The lack of TNF-α in the later stage contributed to the increase of SP-D mRNA after 36 hours.

The changes of SP-D were complicated. LPS is a major ligand for SP-D on *E. coli*. The core region of LPS can be recognized by SP-D[21]. The interaction of SP-D with LPS often results in CRD-dependent bacterial aggregation (agglutination). It is also influenced by the number of repeating saccharide units associated with the terminal O-antigen of the LPS[22]. During this process SP-D directly combines with LPS by means of CRD as well as indirectly participates in the chemotaxis of alveolar macrophage as an opsonin and in the anti-inflammatory response. It also enhances the phagocytosis[23], oxidativeburst and killing ability of neutrophils.

Several mechanisms could possibly be involved in the increase of SP-D expression in the first stage. Cell turnover time is much faster in the process of injury. The first possibility for SP-D over-production is that metabolism of SP-D was altered by the stimulation of LPS. It is estimated that surfactants are recycled at a rate of approximately 10% of the total pool every hour. Increased uptake of SP-D by alveolar type Ⅱ cells could result in an increase of SP-D in lung tissues [14]. The increase of SP-D expression may also partly result from inflammation response induced by the changes of cytokine levels in the LPS model [14]. The synthetical speed of SP-D exceeded the exhausted speed of SP-D. So SP-D was elevated during the first 6 hours and remained at the same level to 12 hours. It was also called compensatory stage.

In the 2nd stage, the down - regulation of SP - D mRNA led to insufficiency of SP-D production due to des-compensations. The interactions of SP-D against LPS together with chemotaxis, phagocytosis, oxidative-burst and killing by neutrophils caused the great over-consumption of SP-D. The up-regulation of SP-D mRNA could not produce enough SP-D within 36-48 hours. SP-D mRNA and SP-D levels reached nadir between 36 and 48 hours when the morphological changes of AT Ⅱ cells and the clinical symptoms were most severe.

In the 3rd stage, the SP-D level rose up due to the accumulation of SP-D mRNA up-regulation. Hyperplasia of AT Ⅱ cells can induce an increased level of SP-D [23]. It has been demonstrated that intratracheal LPS administration can increase the size of type Ⅱ pneumonocyte [24]. Sugahara et al[25] reported that the increased expression of surfactant protein SP-A, SP-B and SP-C was associated with the proliferation of AT Ⅱ cells. In this study the increase of SP-D expression after 48 hours of LPS administration may be contributed to the mechanism similar to Sugahara's report.

In this study the transmission electron microscopy showed that the number of LBs increased at 24 hrs after LPS administration, the size was enlarged at 48 hours and then both in number and size were reduced at 72 hours. The shape and size of LBs at 72 hours were more homogeneous than those of 48 hours. The density of LBs was greatly reduced at 48 hours compared with that 24 and 72 hours. Gigantic LB appeared with the vacuole-like deformity at 48 hours. These changes were coincidentally accompanied by the lowest levels of SP-D mRNA and SP-D. All these pathophysiological and micromorphological changes led to the serious clinical manifestations and even death. This might have resulted from toxication of AT Ⅱ cells and LBs caused by LPS and the exhaustion of pulmonary surfactants. The untrastructural alterations of AT Ⅱ cells and LBs induced by LPS were consistent with SP-D mRNA and SP-D changes despite the fact that SP-D could not be found in LBs[26]. Lamellar body-like and multilamellar forms presented with different densities at different stages. Lamellar bodies were larger in size and were less in number with numerous vesicle-like deformities [17]. The pathological changes showed the damage of AT Ⅱ cells at the early course of LPS-induced ALI.

It can be concluded that the fluctuations of SP-D levels were time-dependent in the early course of ALI induced by LPS. The lowest level of SP-D occurred at 48 hours after LPS administration while the severe multi-deformities of AT Ⅱ cells were presented. A decreased level of SP-D in the lung at the early stage of ALI was associated with a worse clinical outcome. SP-D may be a valuable biomarker in ALI.

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