Skp2 expression exhibits a negative correlation with P27\(^\text{Kip1}\) in lungs of SD rat stress model induced by lipopolysaccharide

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ABSTRACT

In order to investigate action mechanism of Skp2 in SD rat stress model continuously challenged with LPS, not only the histopathological changes but also the distribution and expression of Skp2 and P27\(^\text{Kip1}\) were detected in lungs from SD rats by HE and immunohistochemistry staining respectively. According to present results, Skp2 and P27\(^\text{Kip1}\) mainly distributed in the epithelia of respiratory bronchioles, pseudostratified columnar ciliated epithelia of bronchus and vascular endothelial, a few were located at alveolar wall. As well, area, IOD and IOD/area of Skp2 observably increased in LPS-treated rats \((P<0.05\) or \(P<0.01\), but IOD and IOD/area of P27\(^\text{Kip1}\) apparently decreased in LPS-treated rats \((P<0.05\) or \(P<0.01\)). In conclusion, moderate pathological changes existed in lungs from SD rat stress model challenged by LPS. Moreover, Skp2 high level was negatively correlated with P27\(^\text{Kip1}\) low expression in lungs, it is indicated that Skp2 probably became a key indicator during stress disease process.

Key words: Histological Structure, Lipopolysaccharide, P27\(^\text{Kip1}\), SD Rats, Skp2.

INTRODUCTION

Lung diseases which were brought about by bacteria-mediated infections are an important cause of morbidity and mortality (Hamed et al., 2015). As a major component belonging to the cytoderm of gram-negative bacteria, lipopolysaccharide (LPS) plays a key role in initiating inflammatory response and inducing systemic inflammatory response syndrome and sepsis (Du et al., 2014). LPS can lead to acute lung injury (ALI) and induce acute pulmonary inflammation response, meanwhile, polymorphonuclear neutrophils are activated and accumulated in lung, and oyxradical is released into target organ (Huang et al., 2010). ALI is a clinical syndrome characterized by neutrophil infiltration, diffuse microvascular damage, bronchial and bronchiolar epithelium damage, pulmonary edema and pulmonary fibrosis (Ware and Matthay, 2000; Matthay et al., 2003; Tsushima et al., 2009). Sepsis which are caused by bacterial infection become the main factor of ALI, and animal ALI model is to mimic pulmonary histological structure, morphology and physiological function changes of clinical patients whose endotoxin levels raise (Faffé et al., 2000; Van der Poll and Opal, 2008). Intravenous or intraperitoneal LPS injection has widely been applied in animal ALI model.

Cell cycle is basic biological mechanism (Suzuki et al., 2013) for proliferation of eukaryotic cell which requires sequential activation of cyclin-dependent kinases (CDKs) (Harper, 2001) and CDK inhibitors (CDKIs). SCF(Skp1-Cullin-F-box) complex involves in cell cycle progression and signal transduction, not only influence transcription and post-transcription levels by specific recognition of substrate and subsequent degradation of target protein (especially P27\(^\text{Kip1}\)) via ubiquitination, but are closely related with G1-S phase transformation. A series of biochemical and genetic evidences have shown that SCF\(^{\text{Skp2}}\) can specifically recognize substrate, the activities of which are required for G1 to S transition, S phase and G2 entry (Deshaies, 1999; Nakayama et al., 2004). SCF\(^{\text{Skp2}}\) complex consisting of Skp1, Skp2, Cul1 and Rbx/Roc1 is responsible for recognizing phosphorylated P27\(^\text{Kip1}\) and catalyzing its ubiquitination (Sutterluty et al., 1999; Carrano et al., 2004). During cell cycle progression, the expression levels of Skp2 gene have a periodical change as with many regulators during mitotic time (Wirbelauer et al., 2000). Skp2 expression is low level in quiescent cells and early in G1 phase. Skp2 protein begins to synthesize and accumulate near transition phase of G1 to S, and reaches a high level in S phase, then begins to reduce when cell cycle gets into G2 and M phase (Bashir and Pagano, 2004). Skp2 cooperated with accessory protein Cks1 can specifically recognize and degrade cell cycle inhibitor P27\(^\text{Kip1}\). P27\(^\text{Kip1}\) is metabolically stable and accumulates in quiescent cells, but is rapidly degraded when quiescent cells begin to divide and enter cell division cycle. Moreover, it has been reported that altered expression of Skp2 and P27\(^\text{Kip1}\) are closely related with different levels of malignant tumors (Bloom and Pagano, 2003; Zhang and Wang, 2006), and also can induce hyperplasia of
lipocytes and cause obesity (Cooke et al., 2007). Skp2 contributes to a crucial role in various animal proliferation diseases. This aim was to not only analyze the histopathological changes of respiratory organs but also investigate the expression relationship between Skp2 and P27kip1 in animal’s continuous immune stress process. Study on molecular mechanism of Skp2 shows a certain theoretical value for better understanding the pathogenesis during lung stress disease.

MATERIALS AND METHODS

Experimental animals: Forty healthy SD (Sprague-Dawley) rats of 26 days old and weighing 83±4g were used in present study; these SD rats were purchased from Jiangxi Chinese Medicine College and kept in animal anatomy laboratory of Jiangxi Agricultural University. SD rats were unrestricted access to eat standard chow and drink cooled boiled water, firstly were housed in facility 3 days to adapt environment before this experiment. All experimental procedures were performed in accordance with the guidelines of animal experiments. Forty SD rats were divided into two groups (Twenty SD rats per group) by equalizing body weight (BW) and the variance among groups. SD rats of Saline/D29 group were injected through caudal vein with 0.9% saline (0.2mL). The LPS/D29, LPS/D32, LPS/D35 and LPS/D38 group received single-dose injection of LPS (100µg/kg BW, L2880 Escherichia coli O55:B5, SIGMA) each day for 1, 4, 7 and 10 consecutive days respectively via caudal vein injection. On the 29th, 32nd, 35th, and 38th day, five rats were necropsied at 8h after LPS or saline injection.

Histological evaluation: The lungs were fixed in 4% buffered paraformaldehyde solution for 18-24 h, routinely processed in alcohol-xylene and embedded in paraffin. Then 5 µm sections of lung were made and stained with hematoxylin and eosin (HE) (Zhong et al., 2015). The histological structures of lungs were analyzed under light microscope.

Immunohistochemistry detection: Lung sections were dewaxed with xylene and hydrated with various concentration ethanol solutions according to standard procedure, and incubated for 10 min to block endogenous peroxidase with 3% hydrogen peroxide (H₂O₂). After washing three times with 0.01M PBS (5 min per time), the sections were put in enamelled cup with citrate buffer (pH=6.0) and were heated to 98 °C for 12 min. The sections together with enamelled cup were put in tap water to cool down, and then were applied as the following main procedure: Skp2 (BA2740, Boster co.ltd, Wuhan, China) and P27kip1 (BA0273, Boster co.ltd, Wuhan, China) antibody were added and incubated overnight at 4 °C after blocking 15 min with normal goat serum, then were treated 15 min with HRP-conjugated goat anti-rabbit IgG (SP-9001/9002 link Detection Kits, ZSGB Biotechnology, Beijing, China) at room temperature. These sections were incubated with Horse radish peroxidase conjugated-streptavidin at room temperature for 15 min, and then were colorated 3 min with DAB and counterstained with hematoxylin. These sections were dehydrated in various concentration ethanol solution and xylene, and mounted in neutral gum. Control sections were treated as above steps, but primary antibody was replaced by 0.01M PBS.

Statistical analysis: Immuno-staining section were evaluated under light microscope (BA210; Motic, Xiamen, China), and five non-overlapping fields were collected and measured from per lung section. Skp2 and P27kip1 expression were analyzed with area, IOD (Integrated optical intensity) and IOD/area using digital image analysis software (Image Pro Plus 6.0; Media Cybernetics, Silver Spring, MD) by settings for color and size identification. Graphpad Prism 5 software (San Diego, CA, USA) and SPSS (Version 13.0, Armonk, NY, USA) were used to perform statistical analysis. One-way ANOVA was used for difference comparisons among groups.

RESULTS AND DISCUSSION

Pathological macrostructure and microstructure changes of lungs in stress model: Compared with average LW (Lung weight) of Saline/D29 group, LW of LPS/D32, LPS/D35 and LPS/D38 increase by 9.32%, 11.80% and 12.94% respectively (P<0.05 or P<0.01). No obvious LW change can be found between LPS/D29 and Saline/D29 group. BW (Body weight) in LPS-induced rats from LPS/D29, LPS/D32, LPS/D35 and LPS/D38 group markedly increase than Saline/D29 group (P<0.05 or P<0.01). RLB (Ratio of lung to BW) from Saline/D29 group was significantly lower than LPS/D29 group (P<0.01), however, RLB from LPS/D32, LPS/D35 and LPS/D38 group significantly decrease (<0.01) (Table 1). Furthermore, lung histological structure of Saline/D29 group was intact and clear under light microscope, and there were no exudates in alveolar space. Inflammatory cell infiltration or hemorrhage was also not found in alveolar wall. Conversely, not only diffuse edema in alveolar spaces and lung interstitium existed in LPS group at the 1st day, but also severe inflammatory cell infiltration, hemorrhage and serous-exudation in alveolar space and a thickened interstitial presented too. Alveolar septum thickened in the LPS/D32 group, a number of alveoli and alveolar space decreased or vanished, and bronchus-associated lymphoid tissue (BALT) along a primary bronchiole enlarged. These changes in LPS/D35 and LPS/D38 group were more obvious than LPS/D32 group, BALT were swelling in LPS/D35 rats, and the histological structure integrity of alveoli were destroyed in LPS/D38 rats (Fig. 1).

As common clinical disease, ALI and ARDS mortality rate reach 30%-40% and 50% respectively (Wheeler and Bernard, 2007; The Acute Respiratory Distress Syndrome Network, 2000). ALI model of SD rat induced by LPS is classic stress model (Fujita et al., 1998). In present study, LPS-treated groups get slightly smaller BWs and relatively heavier lungs, but RLB remarkably increased at
Table 1: Analysis on BW, LW and RLB among different SD rat groups injected with LPS and Saline.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lung weight (g)</th>
<th>Body weight (g)</th>
<th>Ratio of lung to BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/D29</td>
<td>0.8766±0.0180</td>
<td>96.448±3.348</td>
<td>9.0896±0.2318</td>
</tr>
<tr>
<td>LPS/D29</td>
<td>0.8950±0.0468</td>
<td>92.759±4.315*</td>
<td>9.6486±0.1468**</td>
</tr>
<tr>
<td>LPS/D32</td>
<td>0.9583±0.0745*</td>
<td>109.415±3.876**</td>
<td>8.7249±0.1581**</td>
</tr>
<tr>
<td>LPS/D35</td>
<td>0.9800±0.0383**</td>
<td>128.850±3.305**</td>
<td>7.6058±0.2720**</td>
</tr>
<tr>
<td>LPS/D38</td>
<td>0.9900±0.0276**</td>
<td>144.213±1.805**</td>
<td>6.8649±0.0610**</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD, compared to Saline/D29 group, **P<0.01 or *P<0.05.

Fig 1: Pulmonary histological changes from Saline/D29, LPS/D29, LPS/D32, LPS/D35 and LPS/D38 group respectively (Magnification 100×). BC: bronchus; PA: pulmonary alveoli; Arrow: thickened alveolar septum; Black triangle: lymphatic tissue hyperplasia.
first and then decreased along with growth. The greater LWs of LPS/D29 group are most likely due to increased collagen deposition that can be detected in histological sections or lung homogenates (Velten et al., 2012). As well, LPS-treated rats are characterized by diffuse edema in alveolar spaces and lung interstitium, severe inflammatory cell infiltration, hemorrhage and serous exudation in alveolar space, and a thickened interbular septa in lung. Thus, lung injury in rats is successfully induced by injecting LPS for consecutive 10 days via caudal vein. Nowadays, domestic animal in large-scale farm are easily challenged by all kinds of negative factors for a long time. Applying continued animal stress model demonstrates great actual significance to investigate pathogenesis, pathological physiology change, clinical diagnosis and drug treatment of lung injury.

**Fig 2: Distribution and expression of Skp2 in lungs from Saline/D29, LPS/D29, LPS/D32, LPS/D35 and LPS/D38 group respectively (Magnification 400×).** Arrow: positive Skp2 cells.

**Immunopositive distribution of Skp2 and P27Kip1 protein in lungs:** According to immunohistochemical analysis, these positive cells were stained yellow brown or dark brown, which varied in shape and size as Fig. 2. In Saline/D29 group, Skp2 positive cells were mainly...
located at the epithelia of respiratory bronchioles, pseudostratified columnar ciliated epithelium of bronchus and vascular endothelial, and a few were found in alveolar wall. However, there are more and color-deeper positive cells in alveolar wall of LPS/ D29, LPS/D32, LPS/D35 and LPS/D38 than Saline/ D29 group (Fig. 2). P27<sup>Kip1</sup> protein mainly distributed in bronchiolar epithelia, few positive cells presented in alveolar mesenchyme. Compared with saline group, there were slightly positive cells in bronchia and less number of reactive cells in alveolar wall after LPS- treatment (Fig. 3).

**Skp2 overexpression promoted degradation of P27<sup>Kip1</sup> level in lungs:** Skp2 distribution area was significantly larger in rat lungs of LPS-treated group than in control group (Fig. 4A). Skp2 IOD from LPS-treated rats (including D29, D32, D35, D38 groups) markedly increased by 17.67%, 30.85%, 24.64% and 16.89% respectively (Fig. 4B). At the same time, average optical density (IOD/area) of Skp2
Fig 4: Skp2 and P27<sup>Kip1</sup> immunohistochemistry staining and relative signal intensities in lungs from different groups. (*P<0.05, **P<0.01).

evidently increased in LPS/D29, LPS/D32, LPS/D35 and LPS/D38 groups (Fig. 4C). However, P27<sup>Kip1</sup> distribution was observably lower in rat lungs from LPS-treated group than in control group (Fig. 4D). Conversely, P27<sup>Kip1</sup> IOD from LPS/D29, LPS/D32, LPS/D35, LPS/D38 groups came down 15.05%, 28.56%, 20.71% and 17.02% respectively (Fig. 4E), and P27<sup>Kip1</sup>IOD/area remarkably decreased respectively as well (Fig. 4F). Obviously, Skp2 expression was induced and P27<sup>Kip1</sup> expression was inhibited in lungs from LPS-induced group. Skp2 and P27<sup>Kip1</sup> play a critical role in cell cycle progression (Polyak <i>et al.</i>, 1994; Weissman, 1997). High Skp2 expression and low P27<sup>Kip1</sup> expression are closely associated with malignant proliferation (Carrano <i>et al.</i>, 1999; Gstaiger <i>et al.</i>, 2001; Shim <i>et al.</i>, 2003), Skp2 depletion in G2 phase or early M phase can bring about continuous accumulation of 4N and 8N DNA within nucleus, and the polyploid cells can’t normally divide into two daughter cells (Nakayama <i>et al.</i>, 2000; Edgar and Orr-Weaver, 2001). LPS can cause lung fibrosis and damage the integrity of rat lung (Cao <i>et al.</i>, 2011). In order to protect lung tissue from LPS damage, animal organism can induce not only helpful division and proliferation of effector cells (Abraham <i>et al.</i>, 2000) but
also synthesis and secretion of inflammatory cytokines to participate in immune stress response. In conclusion, Skp2 expresses high level during malignant or beneficial proliferation, is negatively correlated with P27Kip1 fluctuation as well.

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