Effects of Zinc Finger Protein A20 on Lipopolysaccharide (LPS)-Induced Pulmonary Inflammation/Anti-Inflammatory Mediators in an Acute Lung Injury/Acute Respiratory Distress Syndrome Rat Model

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Source of support:
This work was supported by Medical and Health Scientific Research Foundation of Zhejiang province (2017195219), China

Background:
The aim of this study was to investigate the effects of zinc finger protein A20 on lipopolysaccharide (LPS)-induced pulmonary inflammation/anti-inflammatory mediators in an acute lung injury/acute respiratory distress syndrome (ALI/ARDS) rat model.

Material/Methods:
Forty-eight ALI/ARDS rats were selected and assigned into normal saline (NS) (injected with NS), LPS (injected with LPS), LPS-C1 (injected with pEGFP-C1, NS and LPS), and A20 groups (injected with pEGFP-C1-A20, NS, and LPS). The wet/dry (W/D) ratio of rat lung tissues and total protein concentration and the number of neutrophils in bronchoalveolar lavage fluid (BALF) were detected. Enzyme-linked immunosorbent assay (ELISA) and qRT-PCR were applied to detect the protein and mRNA expressions of A20, IL-10, and TNF-α, respectively. Western blotting was employed to detect the protein expressions of A20, nuclear factor-kappa B (NF-κB) p65 and NF-κB p-P65 in rat lung tissues.

Results:
Compared with the NS group, the W/D ratio of rat lung tissues and total protein concentration and the number of neutrophils in BALF in the other 3 groups increased significantly. The protein and mRNA expressions of A20, IL-10, and TNF-α were significantly higher in the LPS group than in the NS group. The protein and mRNA expressions of A20 and IL-10 were significantly up-regulated and the expression of TNF-α, NF-κB p65, and NF-κB p-P65 was significantly down-regulated in rats injected with A20 compared to those in the LPS group.

Conclusions:
The study provided evidence that zinc finger protein A20 can alleviate pulmonary inflammation by inhibiting TNF-α, NF-κB p65, and NF-κB p-P65 expressions and promoting IL-10 expression.

MeSH Keywords:
Acute Lung Injury • Lipopolysaccharides • Myeloid-Lymphoid Leukemia Protein • Respiratory Distress Syndrome, Newborn

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/901700
Background

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) are life-threatening diseases which are characterized by acute lung inflammation leading to pulmonary congestion, hypoxemia, and decreased pulmonary compliance [1]. ALI/ARDS is the major cause of death in critical care, with a mortality rate of 40–60%, and they also contribute to a long-term illness and disability, with considerable pulmonary and psychological morbidity observed in 50% to 70% of survivors, and only 49% of them can return to employment by 1 year after discharge [2]. ARDS or ALI can be caused not only by primary lung disorders (such as pneumonia, asp...
Inflammatory cells in the interstitial substance and most of the alveolar, alveolar septums were significantly thickened, extravasation of blood in alveolar capillary and extent of edema and hemorrhage in alveolar between 25–50%.

**Table 1.** The degree of pathological change in rat lung tissues.

| Degree | Pathological change                                                                 |
|--------|------------------------------------------------------------------------------------|
| 0      | Normal pulmonary vessels, alveolar, interstitial substance, and bronchial          |
| I      | Pulmonary interstitial edema, a small amount of inflammatory cells, and extent of edema and hemorrhage in interstitial substance and alveolar <25% |
| II     | Inflammatory cells in the interstitial substance and part of the alveolar, alveolar septums were thickened, extravasation of blood in alveolar capillary and extent of edema and hemorrhage in alveolar between 25–50% |
| III    | Inflammatory cells in the interstitial substance and most of the alveolar, alveolar septums were significantly thickened, extravasation of blood in alveolar capillary and extent of edema and hemorrhage in alveolar >50% |

“gene transfection technique for rapid tail vein injection according to hemodynamics” established by Fu Liu et al. [15]. The 4 groups were as follows: the normal saline (NS) group: NS was injected into caudal vein within 5 s (1 mL for each rat); the LPS group: caudal vein of each rat was injected with 1 mL NS within 5 s and was injected with 10 mg/kg LPS (2 mg/ml) (Sigma-Aldrich Chemical Company, St Louis, MO, USA) 8 h later; the LPS-C1 group: 10 μg empty vector pEGFP-C1 and 1 mL NS were injected into caudal vein of each rat within 5 s and was then injected with 10 mg/kg LPS (2 mg/ml) 8 h later. Twenty-four hours after the injection of NS or LPS, the rats in the NS group and rats in other groups were anesthetized with urethane (ethyl carbonate) (7.5 ml/kg). Celiac artery blood was collected and all rats were sacrificed. Samples were collected for follow-up experiments.

Detection of wet/dry (W/D) ratio in rat lung tissues and total protein concentration and the number of neutrophils in bronchoalveolar lavage fluid (BLAF)

After the rats were sacrificed, the thoracic cavity was quickly opened and the right primary bronchus was ligated. The superior lobe of the right lung was removed and put on an electronic balance to measure wet weight, and then was put into an incubator at 60°C for 30 min after cooling to room temperature. A semi-automatic biochemical analyzer (Shanghai Biochemical Analysis Instrument Co., Ltd., Shanghai, China) was used to measure optical density (OD) values per well and calculate the total protein content at the wavelength of 562 nm. To detect the alveolar neutrophil infiltration of rats, BALF was centrifuged at 4°C at 1200 rpm for 10 min, and then the supernatant was discarded. The cells were resuspended in 0.5 mL phosphate-buffered saline (PBS) for precipitation, and 10 μL was collected to count for neutrophils.

**Light microscopy observation**

The inferior lobe of the right lung was quickly removed from killed rats and fixed in 4% poly-formaldehyde for 24 h. The tissues were dehydrated with 70%, 90%, 95%, and 100% graded doses of alcohol. Then, the inferior lobe of the right lung was soaked in cedar oil overnight, washed, and then embedded in paraffin wax. The paraffin block was cut into slices (5 μm), pasted, and toasted at 37°C. Those slices were dewaxed with dimethylbenzene 3 times and dehydrated with graded doses of alcohol. The slices were dried and stained with conventional hematoxylin-eosin (HE) for 10–20 min. Then, the slices were observed under a microscope after dewaxing, dehydration, and mounting. The degree of pathological change was scored (Table 1).

**Enzyme-linked immunosorbent assay (ELISA)**

Left lung tissues (150–200 mg) obtained from killed rats were washed with 4°C pre-cooled NS and dried with absorbent paper. Ophthalmic scissors were used to cut tissues as soon as possible, followed by the addition of 9-fold tissue homogenate medium. An ultrasonic homogenizing machine (Beijing HeDe Biotechnology Co., Ltd., Beijing, China) was used to homogenize tissues for 20 s and refrigerated centrifuge was then used at 3000 rpm at 4°C. The supernatant was identified as lung tissue homogenate and was put into 1.5-mL Eppendorf tubes separately, and preserved in a freezer at –78°C. The whole homogenizing process was carried out on crushed ice. ELISA plates (Shanghai Wu D.-Q. et al.: Zinc finger protein A20 and pulmonary inflammation © Ated Sci Monit, 2017, 23: 3536-3545
Premier sequences for qRT-PCR.

| Gene       | Premier sequence                                      |
|------------|-------------------------------------------------------|
| A20        | Upstream: 5’-GCAGTGTAAAGCCAGGCTAAC-3’                |
|            | Downstream: 5’-TGGGTTTCTCTCTGTAGATTC-3’              |
| IL-10      | Upstream: 5’-AATCCTGCTAACCACCTC-3’                   |
|            | Downstream: 5’-TGGGCTTGTAGACACCTT-3’                 |
| TNF-α      | Upstream: 5’-TACTGAACCTCGGGGTTAGTTCG-3’              |
|            | Downstream: 5’-CAGGCTTGTGCCCTTAGAGAGAC-3’            |
| β-actin    | Upstream: 5’-GATTCGCGTACCCAAGCTTC-3’                 |
|            | Downstream: 5’-GATTGCCACGACATTTGTC-3’                |

qRT-PCR – quantitative real-time polymerase chain reaction.

Alpha Biotechnology Co., Ltd., Shanghai (China) were washed with deionized water 3 times and then dried after soaking in the deionized water overnight. Homogenizing supernatant (10 μL/well) and diluted solution (50 μL/well) were coated at 4°C overnight and then washed with poly (butylene succinate-co-terephthalate) (PBST) 3 times. After the addition of 1% BSA (60 μL/well), the solution was sealed at 37°C for 1 h and then washed with PBST 3 times. Mouse anti-human A20 monoclonal antibody were added into the solution at 75 μL/well with a dilution of 1: 2500 (Active Motif Inc., Carlsbad, CA, USA) at 37°C for 1 h and washed with PBST 3 times. Then, 10 μL sheep anti-mouse IgG-HRP (Beijing North TZ-Biotech Develop., Co. Ltd) was added to each well with a dilution of 1: 7500, incubated at 37°C for 1 h, and washed with PBST 3 times. Each well received 1 drop of color developing solution A and 1 drop of color developing solution B (Shanghai Alpha Biotechnology Co., Ltd., Shanghai, China) and the coloration process lasted for 10 min. The reaction was terminated by adding 2 mol/L H3PO4 (Shanghai Alpha Biotechnology Co., Ltd., Shanghai, China). Color comparator was used to measure the OD450 value. An ELISA kit was used to separate pack IL-10 and TNF-α was purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, China) and we followed the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Lung tissues (about 100 mg) were cut in a plate placed on ice and 1 mL extracting RNA (TRIZOL reagent, Invitrogen Inc., Carlsbad, CA, US) was added and performed with repeated aspiration to obtain an emulsion. After the addition of 200 μL chloroform, the solution was quickly shaken for 15 s and placed at room temperature for 5 min. The tissue samples were then centrifuged at 13 000 rpm at 49°C for 15 min and the obtained supernatant was added to 500 μL dimethyl carbinol and precipitated on ice for 1 h. The supernatant was discarded and the precipitation was washed with 75% alcohol and the centrifugation was repeated. Finally, moderate diethyl phosphorocyanidate (DEPC) was used to dispose the precipitation of water-soluble RNA and extract total RNA. cDNA was obtained from 20 μL reverse transcription reaction system. The cDNA (3 μL), DEPC water, buffer solution, dNTPs, Taq DNA polymerase, and primers were then added into the 25 μL reaction system for reaction in PCR amplification instrument (USA model: PTC-100TM, MJ Research Inc., Watertown, MA). Amplification conditions were as follows: denaturation at 94°C for 3 min, annealing at 56°C for 30 min, extension at 72°C for 8 min, and cycling 35 times. PCR products were conducted with 2% agarose gel electrophoresis and ID image analysis software (Eastman Kodak Company, Rochester, NY, USA) was applied for the band analysis. Relative expressions of mRNA of detected specimens = the absorbance of amplified products to be tested/the absorbance of amplified products obtained from the β-actin-specific primers. The relative quantity of target genes was 2−ΔΔCt [16]. ΔCt = the domain value of the target gene – the standard group (CT-target – CTβ-actin) – the group to be tested (CT-target – CTβ-actin). The primer sequences of A20, TNF-α, IL-10, and β-actin were shown in Table 2.

Western blotting

Lung tissue homogenate were taken out for the detection of total protein concentration using a BCA Protein Quantitation kit (Pierce, Rockford, IL). Based on the samples with minimum concentration, other samples were adjusted to the same concentration and 5×SDS sample buffer of the same volume were added. The samples were mixed using vortex oscillation and denaturation at 100°C for 5–10 min. Then, the samples were conducted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membrane, sealed, and incubated with primary antibody (Rabbit anti-mouse A20, nuclear factor-kappa B (NF-kB) p65, NF-kB p-P65 and GAPDH antibody, R&D Systems, Minneapolis, MN, USA), at 4°C overnight.
After membrane cleaning, the samples were incubated with secondary antibody at room temperature for 1 h. Then, the cleaning was performed for 10 min repeated 3 times, and detected by electrogenerated chemiluminescence (ECL).

Statistical analysis

Results are displayed as mean ± standard deviation ($\bar{x} \pm s$). All data were analyzed by SPSS 21.0 software (SPSS, Inc, Chicago, IL, USA). One-way analysis of variance (ANOVA) was utilized to compare data among multiple groups, and the comparisons between 2 groups were conducted by Student's t-test ($P<0.05$ set as a standard to show significance). $P<0.05$ represented that the difference is significant.

Results

Construction and identification of vector pEGFP-C1-A20

pUC57-A20 plasmid was obtained after the connection of PCR product of zinc finger protein A20 gene and pUC57 plasmid. Double-enzyme digestion was performed on the recombinant plasmid, vector pUC57 was removed, and a 573 bp insertion sequence, which was in accordance with the design fragment, was obtained. Then, the A20 target fragment was connected with vector pEGFP-C1 to construct the recombinant pEGFP-C1-A20 plasmid, and the actual length was in accordance with the design length (Figure 1).

Comparisons of the W/D ratio in rat lung tissues and total protein concentration and the number of neutrophils in BLAF among 4 groups

The W/D ratios of rat lung tissues indicate the lung water content. The results were as follows. Compared with the W/D ratio of rat lung tissues in the NS group (4.30±0.41), the W/D ratio in the LPS group (5.58±0.53), the LPS-C1 group (5.55±0.52), and the A20 group (4.87±0.46) were clearly increased (all $P<0.05$); however, the W/D ratio in the A20 group declined significantly more than in the LPS group ($P<0.05$). Total protein concentration in BLAF was also detected to reflect the alveolar-capillary membrane permeability. The total protein concentration in BLAF in the LPS group (348.50±17.42), the LPS-C1 group (345.18±17.26 μg/ml) and the A20 group (315.31±15.76) increased markedly more than in the NS group (214.22±10.71); however, the total protein concentration in BLAF in the A20 group was clearly down-regulated compared to the LPS group ($P<0.05$). The alveolar neutrophil infiltration was also detected, and the results were as follows: compared with the NS group (28.00±5.46), the LPS group (326.00±11.75), the LPS-C1 group (295.50±17.71) and the A20 group (161.91±14.48) had elevated neutrophil counts, while compared with the LPS and LPS-C1 groups, the A20 group had significantly down-regulated neutrophil counts ($P<0.05$) (Figure 2).

Comparisons of HE staining of rat lung tissues after injection among 4 groups

The pathological changes of rat lung tissues were observed. The alveolus of the rats in the NS group was complete, and there was no edema in the alveolar wall and no obvious inflammatory cell infiltration in the lung. No significant difference between the LPS group and the LPS-C1 group was observed, and in both groups alveolar septums were thickened, and inflammatory cell infiltration and red blood cell and neutrophil exudation at the alveolar cavity were observed; a large number of red blood cell and neutrophil exudation at the cell gap of lung tissues were observed; the degree of inflammation in the A20 group was lower than that in the LPS and LPS-C1 groups, edema and inflammatory infiltration of lung tissues also decreased in the A20 group (Figure 3). The degree of pathological change in rat lung tissues was scored and is shown in Table 3.
Figure 2. Comparisons of W/D ratio in rat lung tissues and total protein concentration and the number of neutrophils in BLAF among 4 groups. (A) W/D ratio in rat lung tissues; (B) total protein concentration in BLAF (μg/mL); (C) the number of neutrophils in BLAF; BLAF – bronchoalveolar lavage fluid; NS – normal saline; LPS – lipopolysaccharide; C1 – pEGFP-C1; ^ P<0.05 compared with the NS group; # P<0.05 compared with the LPS group; Experimental data are presented as mean ± standard deviation (SD); n=12.

Figure 3. HE staining images of rat left lung tissues 24 h after injection with NS or LPS among 4 groups (400× magnification). NS – normal saline; LPS – lipopolysaccharide; C1 – pEGFP-C1; (A) the NS group; (B) the LPS group; (C) the LPS-C1 group; (D) the A20 group; n=12.
Comparisons of zinc finger protein A20 expression in rat lung tissues after injection among 4 groups

Compared with the NS group, the expression of A20 was significantly higher in the LPS group, the LPS-C1 group, and the A20 group (all P<0.05), indicating that LPS could induce the expression of zinc finger protein A20 in lung tissues. The expression of A20 was significantly elevated in the A20 group compared to the LPS group and the LPS-C1 group (P<0.05), indicating that vector pEGFP-C1-A20 was successfully transfected into the experimental rats to synthetize zinc finger protein A20. No significant difference was found in the expression of A20 between the LPS group and the LPS-C1 group (P>0.05) (Figure 4).

Comparisons of IL-10 and TNF-α expressions in rat lung tissues after injection among 4 groups

IL-10 expression in rat lung tissues increased significantly in the LPS group, the LPS-C1 group, and the A20 group compared to the NS group (P<0.05), and the A20 group presented

Table 3. The degree of pathological change in rat lung tissues.

| Degree | N  | O  | I  | II | III | Ridit value |
|--------|----|----|----|----|-----|-------------|
| NS group | 12 | 12 | 0  | 0  | 0   | 0           |
| LPS group | 12 | 0  | 4  | 8  | 0   | 0.83*       |
| LPS-C1 group | 12 | 0  | 5  | 7  | 0   | 0.88**      |
| A20 group | 12 | 0  | 7  | 5  | 0   | 0.86**      |

* P<0.05 compared with the NS group; # P<0.05 compared with the LPS group; N – number; NS – normal saline; LPS – lipopolysaccharide; C1 – pEGFP-C1.
significantly higher expression of IL-10 than in the LPS group and the LPS-C1 group \((P<0.05)\), indicating that high expression of A20 could up-regulate the release of IL-10 to a certain degree. The expression of TNF-\(\alpha\) in rat lung tissues was significantly lower in the NS group compared with that in the LPS, LPS-C1, and A20 groups \((P<0.05)\); the expression of TNF-\(\alpha\) in rat lung tissues declined markedly in the A20 group compared to the LPS group and the LPS-C1 group, indicating that high expression of A20 could down-regulate the release of TNF-\(\alpha\) to a certain degree (Figure 5).

Comparisons of mRNA expressions of A20, TNF-\(\alpha\), and IL-10 in rat lung tissues after injection among 4 groups

The mRNA expression of A20 was significantly elevated in the LPS group, the LPS-C1 group, and the A20 group compared to the NS group; the A20 group exhibited higher mRNA expression of A20 than the LPS group and the LPS-C1 group \((P<0.05)\) (Figure 6A). The mRNA expression of IL-10 was significantly up-regulated in the LPS, LPS-C1, and A20 groups compared to the NS group; the A20 group showed increased expression of IL-10 compared to the LPS group and the LPS-C1 group \((P<0.05)\) (Figure 6B). The mRNA expression of TNF-\(\alpha\) increased significantly in the LPS group, the LPS-C1 group and the A20 group compared to the NS group; the A20 group presented decreased expression of TNF-\(\alpha\) compared to the LPS group and the LPS-C1 group \((P<0.05)\) (Figure 6C).

Comparisons of protein expressions of A20, NF-\(\kappa\)B p65, and NF-\(\kappa\)B p-P65 in rat lung tissues among 4 groups

The A20 group exhibited significantly up-regulated A20 expression compared to the NS, LPS, and LPS-C1 groups, and down-regulated NF-\(\kappa\)B p65 and NF-\(\kappa\)B p-P65 expression compared to the LPS and LPS-C1 groups, indicating that the high protein expression of A20 can inhibit the expression of NF-\(\kappa\)B p65 and NF-\(\kappa\)B p-P65 to some extent, thus influencing the NF-\(\kappa\)B signaling pathway (Figure 7).

Discussion

ALI/ARDS are syndromes of acute respiratory failure which occur as a series of malignancy attributed to diverse risk factors [17]. In spite of the advances in therapeutic principles and surgical techniques, ALI/ARDS still have high incidence and poor outcome, and ALI/ARDS continues to be a leading cause of morbidity and mortality in humans [18]. A20, known for many years as an anti-apoptotic protein through inhibiting the NF-\(\kappa\)B signaling pathway, received attention in recent studies as an important negative regulator of inflammation [19]. Therefore, the present study aimed to elucidate the role and mechanism of A20 in pulmonary inflammation in an ALI/ARDS rat model.
Initially, our study revealed that the W/D ratio of rat lung tissues and total protein concentration and the number of neutrophils in BALF were significantly reduced in the A20 group compared to the LPS group. LPS is one of the main components of gram-negative bacteria cell walls, and upon infection with LPS, an activation of the inflammatory cascade leading to the release of inflammatory mediators is involved in the occurrence and progression of ALI/ARDS [20]. The W/D ratio was calculated as an indicator of lung edema, and LPS has been reported to be able to elevate the total protein concentration in BALF; however, A20 can inhibit the increased concentration of inflammatory cells in BALF induced by LPS through down-regulating the NF-κB signaling pathway, so the W/D ratio of rat lung tissues and total protein concentration in BALF were significantly decreased in the A20 group compared to the LPS group [13,21]. Neutrophil infiltration into the alveolar space causes lung injury; hence, neutrophil depletion plays a protective role in ALI [22]. Previous research also demonstrated that activation of NF-κB reduces neutrophil apoptosis of lungs in ALI patients [23]. The possible mechanism by which LPS induces ALI/ARDS is that LPS activates the NF-κB signaling pathway to increase the expressions of many cytokines, including TNF-α [24]. However, A20 has been extensively reported to be a potent inhibitor of activation of the NF-κB signaling pathway. A20 regulates TNF-mediated NF-κB activation by functioning as a dual ubiquitin-editing enzyme. TNF and LPS trigger the phosphorylation of A20 at the site of Ser181, which, by a currently undetermined mechanism, improves the ability of A20 to inhibit the NF-κB signaling pathway [25]. Another laboratory study revealed that A20 null mice fail to block TNF-induced NF-κB activation, develop serious inflammation, and die quickly, indicating the important role of A20 in anti-inflammatory processes [26]. Therefore, it is reasonable to speculate that LPS increases the expression of A20, and the accumulated expression of A20 results in the blockade of NF-κB signaling pathway, repressed sensitivity of LPS in rats, and alleviated progression of ALI/ARDS.

In addition, we found that the protein and mRNA expressions of A20, IL-10, and TNF-α were significantly elevated in the LPS group compared to the NS group. It is becoming increasingly evident that inflammatory mediators play important roles in the pathogenesis and development of ALI/ARDS; therefore, the identification of the key cytokines in ALI/ARDS coupled with the investigation of potent inhibitors would make it possible to develop anti-inflammatory therapy with significant clinical efficacy [27]. TNF-α is known as an elevated pro-inflammatory cytokine in ALI/ARDS that can stimulate the production of other cytokines, while IL-10 is a highly potent anti-inflammatory cytokine that can prevent macrophages from producing cytokine, and the ratio of concentrations of TNF-α/IL-10 has been reported to be significantly increased in ALI/ARDS patients [28].

Furthermore, our study demonstrated that the high protein expression of A20 can inhibit the expression of NF-κB p65, thus influencing the NF-κB signaling pathway. Our previous research has clearly established the role of A20 in the negative regulation of inflammation via suppressing the activity of the NF-κB signaling pathway, resulting in the downregulation of pro-inflammatory TNF-α and the up-regulation of anti-inflammatory IL-10 [29]. The balance between pro- and anti-inflammatory cytokines in the lungs of ALI/ARDS patients is a key step toward understanding the pathogenesis of the devastating disease and helping in the development of clinical strategies to reduce the clinical severity and mortality of ALI/ARDS, and A20 is a potent regulator of this balance [28].

Conclusions

In conclusion, our study clarifies that LPS, IL-10, TNF-α can increase the expression of A20 in a rat model, and the elevated expression of A20 can inhibit the sensitivity of LPS in rats and alleviate ALI/ARDS. Moreover, A20 represses inflammation by promoting the expression of anti-inflammatory IL-10 and inhibiting the expression of inflammatory TNF-α. Therefore, enhancing the expression of A20 may be a promising therapeutic strategy in treating inflammatory autoimmune diseases. However, the effects of A20 on other organs and other inflammatory mediators have yet to be clarified.

Competing interests

None.

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