Caspase-1 inhibitor reduced the lung injury in a mouse model of pleuropneumonia caused by *Actinobacillus pleuropneumoniae*

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Abstract

The pathogenesis of porcine contagious pleuropneumonia is poorly understood. In the present study, a mouse model of intranasal infection by *Actinobacillus pleuropneumoniae* (App) was used to examine lung inflammation. The pathological results of lung tissues showed that App-infected mice showed dyspnea and anorexia, with severe damage by acute hemorrhage, and infiltration of eosinophils and lymphocytes, as well as increased expression of caspase-1 p20, interleukin (IL)-1β, IL-6, IL-8, IL-18 and tumor necrosis factor (TNF)-α. Caspase-1 inhibitors reduced both lung tissue damage and the expression of caspase-1 p20, IL-1β, IL-6, IL-8, TNF-α and IL-18 in infected mice. These findings suggest that the caspase-1 dependent pyroptosis involved in the pathogenesis of the mouse pleuropneumonia caused by *App* and the inhibition of caspase-1 reduced the lung injury of this pleuropneumonia.

Key words: porcine contagious pleuropneumonia, mice, caspase-1 inhibitors, caspase-1 dependent pyroptosis

Introduction

*Actinobacillus* is known as both a primary and secondary pathogen (Czyżewska-Dors et al. 2017). Porcine contagious pleuropneumonia (PCP), induced by *App* infection, is a very serious respiratory disease with high infectivity and fatality since the lung injury in this disease is difficult to prevent and treat. It causes lowered productivity and economic loss in the pig industry in many countries (Pereira et al. 2018, Sassu et al. 2018). Its detailed pathologic mechanisms are still unclear (Sassu et al. 2018).

Through many kinds of pattern recognition receptors (PRRs), hosts can identify distinct pathogen-associated molecular patterns (PAMPs) present in microorganisms. Identification of microbial pathogens is mediated by PRRs, such as nucleotide-binding oligomerization domain-like receptors (NLRs). After the NLRs recognize microbial PAMPs, they activate inflammasomes to mediate the production of proinflammatory cytokines (Paramel et al. 2015). Meanwhile, following inflammasome pathway activation, procaspase1 is processed into two cleaved subunits called p10 and p20 (Miao et al. 2011). Subunit p20 cleaves the precursors...
of the IL-1β and IL-18 to mature IL-1β and IL-18 (van de Veerdonk et al. 2011, Oviedo-Boyso et al. 2014, Compan et al. 2015). The mature IL-1β and IL-18 further lead to a proinflammatory form of programmed cell death, namely, pyroptosis (Qiu et al. 2017). Hu et al. (Hu et al. 2015) reported that NLRP3 expression elevated in App-infected mice. Some studies revealed overproduction of proinflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, IFN-γ (Huang et al. 1999, Gomez-Laguna et al. 2014, Kowalczyk et al. 2014, Czyzewska-Dors et al. 2017, Li et al. 2018). Therefore, we hypothesized that caspase-1 dependent pyroptosis was involved in the pathogenesis of pleuropneumonia caused by App.

Ac-YV AD-CMK (Ac-Tyr-Val-Ala-Asp-CMK) is a potent and irreversible inhibitor of caspase-1 and displays anti-inflammatory and anti-pyroptotic effects. There is no evidence that pharmacological caspase-1 inhibition reduces the lung injury of mouse pleuropneumonia caused by Actinobacillus pleuropneumonia. In this study, we detected pharmacological effects of caspase-1 inhibitor in an intranasally App-infected mouse model.

Materials and Methods

Bacterial strains and culture conditions

App serotype I (Hu et al. 2015) strain was grown in Trypticase Soy Broth (TSB) (Qingdao Rishui biotechnology Co. Ltd. China) supplemented with 15 μg/ml beta-nicotinamide adenine dinucleotide trihydrate-1 (Beijing Dingguo Changsheng Biotechnology Co. Ltd. China) and 10% newborn calf serum (Gibco, Burlington, VT) at 37°C in 5% CO₂.

Establishment of animal models with infection

All animal procedures were performed according to protocols approved by the Biological Studies Care and Use Committee of Hunan Province, P.R.C. Thirty 4-week BALB/C mice (from Hunan SJA Lab Animal) were divided equally into a control group, an infected group, and an inhibitor treated group (n=10 in each group). Mice in infected group and caspase-1 inhibitor treated group were intranasally inoculated with 0.25mL diluent containing 1×10⁸ CFU/mL (10×LD50) App (Hu et al., 2015). Caspase-1 inhibitor Ac-YVAD-CMK (in PBS containing 1% DMSO, from Shanghai Mocell Biotechnology Co. Ltd. China) was tail vein injected at a dose of 6.5 mg/kg 1 h before intranasal App inoculation in the caspase-1 inhibitor treated group. Mice in the control group were intranasally inoculated with an equal volume of normal saline (0.9% wt/vol NaCl). The mice were executed at 48 hrs after post-inoculation.

The right lungs of the mice were collected for RNA extraction (in liquid nitrogen), protein extraction (frozen at -80°C) and histopathological analysis. The left lungs were used for measuring the wet weight (W) and the dry weight (D). The left lungs for measuring dry weight were desiccated in an oven at 65°C for 48 h. The water content was obtained by calculating the W/D weight ratio.

Histopathological observation

Histopathological analysis was performed using routine methods. In brief, the lungs were fixed in 10% neutral buffered formalin for 24 h, processed routinely, and embedded in paraffin wax. Tissue sections (3-4 μm) were stained with haematoxylin and eosin (HE).

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Table 1. Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers (5’→3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-action</td>
<td>F: 5’ TTCCTTCTCTGGGTATGGAAT3’</td>
<td>178 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GAGCAATGATCTTGATCTTC3’</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: 5’ GCCTCGTGCTGTCGGACCCA 3’</td>
<td>199 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TCCAGCTGAGGGTGTTGT 3’</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>F: 5’ GCTGTTGACAACCCAGGGGCT 3’</td>
<td>107 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ AGGCTCCAGTCGGTAAGGTGT 3’</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>F: 5’ GCACCCACTTCCAGTCG GC 3’</td>
<td>217 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ CATGCTCTCTGTCGGGCG 3’</td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>F: 5’ ATGCTTTCTGAGCTTCTGCC 3’</td>
<td>168 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ ATGCCCTGGGGCCAAGAGG 3’</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5’ ATCCGCGACGTGGAACTGGC 3’</td>
<td>159 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TTGGGACCGACACCCCGA 3’</td>
<td></td>
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</tbody>
</table>
Caspase-1 inhibitor reduced the lung injury of mouse...

Real-time quantitative PCR analysis

Oligonucleotide primers: The sequences of oligonucleotide primers in this study were designed using Primer 5 software and are listed in Table 1. Primers for these selected genes were designed based on mouse sequences obtained from the National Center for Biotechnology Information (NCBI). They were synthesised at Shanghai biotechnology Co. Ltd.

Total RNA extraction: A piece of lung tissue was snap frozen in liquid nitrogen. RNA was extracted using Trizol (TaKaRa Biotechnology (Dalian) Co. Ltd. China) according to the manufacturer's instructions. The concentration of total RNA was measured using spectrophotometry.

cDNA synthesis: 1 μg total RNA was transcribed into cDNA using 5×TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (AT341) (Beijing TransGen Biotech Co. Ltd. China). Briefly, the total volume of the reaction was 20 μl, consisting of 1 μg total RNA, 4 μl 5×TransScript All-in-One SuperMix for qPCR, 1 μl gDNA Remover, adding RNase-Free ddH₂O to 20 μl. Samples were incubated at 42°C for 15 min, followed by 85°C for 5 s to inactivate TransScripRT/TI and gDNA Remover.

Real-time quantitative PCR amplification: Real-time PCR amplification was performed using 2×TransStartTip Green qPCR Super Mix (AQ141) (Beijing TransGen Biotech Co. Ltd. China). Briefly, the total volume of the reaction was 20 μl, consisting of 1 μl template cDNA, 1 μl forward Primer (10 μM), 1 μl reverse Primer (10 μM), 10 μl 2×TransStart Tip Green qPCR Super Mix, adding ddH₂O to 20 μl.

The reactions were carried out as an initial pre-incubation at 94°C for 30 seconds, followed by 40 amplification cycles of: 94°C for 5 s, 60°C for 30 s. Melting curve analysis was performed immediately after amplification from 65 to 95°C with continuous fluorescence acquisition.

Calculation of relative gene expression: The level of gene expression in the present study was analyzed...
using the “Delta-delta method” for relative quantification. The expression of the selected gene was normalized to that of the reference β-action gene using the equation 2^−ΔΔCq = 2^−ΔCq(sample - ΔCq control) and further converted to relative mRNA expression (Hu et al. 2015).

**Western blot analysis**

Total protein was extracted from the lung tissues (Zhao et al. 2015). The protein concentration of the lung tissues was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Twenty to thirty μg of each sample was analyzed by Western blot analysis. The suspension was subjected to 10% acrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto a nitrocellulose filter. After blocking with 5% (w/v) non-fat milk dissolved in PBS for 2 h, the membranes were incubated at 4°C overnight with primary antibodies of IL-1β, IL-18, NLRP-3, caspase-1, caspase-1p20 and β-actin (1:1000) (Wuhan Boster Biotechnology Co., Ltd. China), followed by incubation with HRP labeled goat anti-mouse IgG (1:1000) (Wuhan Boster Biotechnology Co. Ltd. China) for 1 h. Western blotting bands were quantified using BioRad Software.

**Statistical analysis**

Statistical analysis was performed using one-way analysis of variance and post-hoc tests. The error bars represent the standard error of means p<0.01 values were considered significant.

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Fig. 2. Caspase-1 inhibitor markedly increased IL-1β, IL-18 expression in the lung of App-infected mice. (A) Caspase-1, caspase-1 p20 protein levels detected by western blot in lungs of mice (** p<0.01), (B) IL-1β, IL-18 protein levels detected by western blot in lungs of mice (** p<0.01)

Fig. 3. Caspase-1 inhibitor decreased some preinflammatory factor expression in pulmonary lesion (** p<0.01)
Caspase-1 inhibitor reduced the lung injury of mouse pleuropneumonia

Results

Caspase-1 inhibitor reduced lung injury caused by App infection

Histopathological findings of the mouse lung tissue are shown in Fig. 1A, B, C. The pulmonary organizational structure of the control group was normal. In the infected group, significant pulmonary inflammation, hemorrhage, interstitial edema, and inflammatory cells were observed in most alveolar spaces. The amount of inflammatory cell infiltration and alveolar edema were reduced in the caspase-1 inhibitor treated group when compared with the infected group. To further evaluate the lung injury, the pulmonary W/D weight was assessed (Fig. 1D). Consistent with histological changes, pulmonary W/D weights in the infected group were much higher than those in the control group (p<0.01) and significantly decreased in the caspase-1 inhibitor treated group (p<0.01).

Caspase-1 inhibitor markedly decreased the expression of caspase-1 p20, IL-1β, IL-18 in the lung of intranasally App-infected mice

Caspase-1 p20, IL-1β, IL-18 were markedly increased in the infected group compared with the control group (p<0.01; Fig. 2A, B). However after Caspase-1 inhibitor treatment, caspase-1 p20, mature IL-1β, mature IL-18 were markedly decreased. These data demonstrate that caspase-1 dependent pyroptosis play a key role in the pathogenesis of pleuropneumonia caused by App.

Caspase-1 inhibitor decreased the expression of some preinflammatory factors in pulmonary lesion

The mRNA expression levels of some preinflammatory factors (IL-1β, IL-6, IL-8, IL-18 and TNF-α) were measured in the lung tissues. The Caspase-1 inhibitor significantly reduced IL-1β, IL-6, IL-8 and TNF-α expression levels in lung tissues (p<0.01; Fig. 3).

Discussion

In the present study mice showed dyspnea and anorexia after intranasal inoculation with App at post-inoculation 48 h. In the autopsy, histopathological results indicated that the lungs were found to be severely damaged by acute hemorrhagic pneumonia along with the following characterizations: hemorrhage, eosinophils, and lymphocyte infiltration.

Pyroptosis is a type of caspase-1 dependent inflammatory cell death pattern with cell lysis. Activated caspase-1 transforms inactive pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18 (Yu et al. 2014). The mature IL-1β and IL-18 then further induce pyroptosis. The formation of cell membrane pores in the pyroptosis leads to the release of many cell contents, which arouses a strong inflammatory response (Vande Walle and Lamkanfi 2016). Chen et al. (1996) first reported that caspase-1-dependent pyroptosis was found in mouse macrophages infected with the gram-negative bacteria Shigella flexneri. A growing body of evidence suggests that caspase-1-mediated pyroptosis may take part extensively in inflammatory processes and bacterial diseases (Danelishvili et al. 2013). In this study, it was found that expressions of caspase-1p20, IL-1β, IL-18 were up-regulated in a mouse pleuropneumonia model induced by App infection. Furthermore, caspase-1 inhibitor could reduce pulmonary tissue damage and expressions of IL-1β and IL-18.

Cytokines play a critical role in infectious diseases (Degre 1996, Imanishi. 2000). Previous works revealed that overproduction of proinflammatory cytokines in the lung was an essential feature of App infection. These proinflammatory cytokines include: IL-1β, IL-6, IL-8, TNF-α, IFN-γ (Gomez-Laguna et al. 2014, Kowalczyk et al. 2014, Czyzewska-Dors et al. 2017, Li et al. 2018). Huang et al. (1999) found that TNF-α and IL-1 levels were positively correlated with the severity of acute disease. IL-1β, IL-6 and IL-8 could directly and indirectly induce an inflammatory response in mice infected by App. Hsu et al. (2016) confirmed that the overproduction of proinflammatory cytokines induced pulmonary lesions in PCP. In this study, it was found that pro-inflammatory cytokine expression of IL-1β, IL-8, IL-18, IL-6 and TNF-α was increased in a mouse pleuropneumonia model induced by App infection. After caspase-1 inhibitor treatment, however the expression of IL-1β, IL-8, IL-18, IL-6 and TNF-α was significantly decreased. All the above findings support the hypothesis that caspase-dependent pyroptosis is involved in the pathogenesis of pleuropneumonia caused by App, and caspase-1 inhibitor can reduce the lung injury of mouse pleuropneumonia.

Acknowledgements

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References


